THE ROLE OF KERATINOCYTE ALPHA(V) BETA-6 INTEGRIN IN THE REGULATION OF TRANSFORMING GROWTH FACTOR-BETA DURING WOUND HEALING

by

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M.D., Tehran Azad Medical School, 2000

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Craniofacial Science)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

October, 2010

Abstract

The stratified squamous epithelium of skin and mucosa is mostly formed from keratinocytes and protects the organism from its surrounding environment. Any damage to this protective layer is restored through re-epithelialization – an essential part of wound healing. Therefore, studying the regulatory mechanisms of keratinocytes during re-epithelialization is important in understanding the processes involved in both normal and impaired wound healing.

The keratinocyte αvβ6 integrin is induced during wound healing. Interestingly, αvβ6 integrin can activate both fibrogenic transforming growth factor-β1 (TGF-β1) and anti-fibrogenic TGF-β3. The role of αvβ6 integrin, however, especially in regards to the regulation of TGF-βs, during wound healing, is largely unknown.

In the present study, we investigated the potential for the αvβ6 integrin-mediated regulation of TGF-β1 and TGF-β3 during wound healing in gingival and skin wound tissue sections. Furthermore, we investigated the possible regulatory mechanisms of TGF-β1 activity by αvβ6 integrin in keratinocytes.

Spatio-temporal co-accumulation of αvβ6 integrin with TGF-β1 and TGF-β3 in the wound epithelium suggested that αvβ6 integrin may locally regulate both isoforms during wound healing. Prolonged co-expression of αvβ6 integrin and TGF-β3 in the scar-free gingival wound epithelium may potentially have an important role in the protection of gingiva from scarring.

Our *in vitro* data showed that keratinocytes responded differentially to low levels of endogenously produced TGF-β1 compared to high amount of extracellular matrix (ECM)-bound latent TGF-β1. While the data were suggestive of activating endogenous TGF-β1 by the keratinocyte αvβ6 integrin, high levels of ECM-associated TGF-β1 was removed by keratinocytes in the presence of αvβ6 integrin allowing them to overcome the TGF-β1-mediated inhibitory effects on the cell proliferation.

Taken together, our data showed a potential for the αvβ6 integrin-mediated local regulation of TGF-β1 and -β3 during wound healing. Prolonged expression and co-accumulation of αvβ6 integrin and TGF-β3 may be important in the protection of gingival wounds from scar formation. Our finding of αvβ6 integrin-mediated removal of the matrix-bound TGF-β1 in keratinocytes may be important at the time of wound closure, when keratinocytes typically show increased proliferation rate despite high levels of TGF-β1 in the surrounding matrix.

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Co-authorship Statement

The chapters presented in this thesis have been prepared by the candidate with the guidance and supervision of Dr. Hannu Larjava and Dr. Lari Häkkinen. Chapters 2 and 3 of this dissertation include a published and a submitted manuscript, respectively. The contribution of the co-authors in each manuscript is as follows:

Chapter 2: Dr. Hannu Larjava and Dr. Lari Häkkinen participated in the collection of human gingival unwounded and wound tissue samples. Drs. Larjava, Häkkinen, Gallant-Behm, Hart and Wiebe participated in the collection of pig wound tissue samples. Dr. Gardner assisted in the transcriptional profiling of gingival samples. Dr. Honardoust helped with some immunostainings. The manuscript was reviewed by all the co-authors.

Chapter 3: Dr. Häkkinen isolated the progenitor-like mouse keratinocytes. Dr. Koivisto participated in the FACS analysis and mouse keratinocyte proliferation assays. Dr. Jiang helped with the real-time PCRs. Dr. Owen participated in immunohistochemical analysis. Hanah Tsao helped with some of the ELISA. All the co-authors participated in the revising of the manuscript.

To:

My family

Acknowledgement

I would like to express my sincere gratitude to my advisor, Dr. Hannu Larjava, for his invaluable guidance, caring, and understanding through my years of studying. I would also like to offer my deepest appreciation to my co-advisor, Dr. Lari Häkkinen, who has been always there to listen and give advice. I'm also truly grateful to the other members of my advisory committee Dr. Aziz Ghahary, Dr. Edward Putnins and Dr. Dieter brömme for their valuable advice and critical comments on this dissertation.

Collective and individual acknowledgments are also owed to my colleagues in the Laboratory of Periodontal Biology. I would especially like to record my gratitude to Dr. Leeni Koivisto, Dr. Guoqiao Jiang, Dr. Gethin Owen, and Dr. Dariush Honardoust for their contribution to my research and for sharing their ideas with me, answering my questions, and their friendship. I cannot thank enough Mr. Cristian Sperantia for his excellent technical assistance, his joyful presence, caring nature and friendship.

My continuation in this Ph.D. program couldn't have happened without the enduring love and support provided by my parents and my husband, Shahab. We earned this degree together as a family.

Chapter 1: Review of Literature

1.1. Skin and mucosa

Skin and mucosa, including the mucosa of oral cavity, are mainly composed of a stratified squamous epithelium and a connective tissue, which are called epidermis and dermis in skin, respectively (Gawkrodger, 2002; Kirfel and Herzog, 2004; Morasso and Tomic-Canic, 2005). Epithelium and connective tissue are separated from each other by the basement membrane (BM), a thin specialized layer of extracellular matrix (ECM) which is rich in laminin and type IV collagen (Kirfel and Herzog, 2004). The epithelium as the outermost barrier layer of the skin and mucosa is mainly composed of multi layers of keratinocytes and serves as body's first line of defense from the surrounding environment (Kirfel and Herzog, 2004). The basal cell layer of epidermis and mucosal epithelium contains the stem cells with lifelong cell division ability. Stem cells give rise to transit-amplifying cells which retain the ability of cell dividing for 3 to 5 times before turning into committed cells that move upward from the basal layer to finally go through terminal differentiation (Kirfel and Herzog, 2004). Under normal conditions keratinocytes are interconnected through desmosomes, and firmly anchored to the basement membrane via hemidesmosomes (Kirfel and Herzog, 2004). Stratified epithelium of the epidermis and the gingival and hard palate tissue of the oral cavity forms a non-viable keratinized layer which considerably adds to the protective role of those epithelia (Presland and Jurevic, 2002). The major cells of the dermis and mucosal connective tissue are fibroblasts which are responsible for secreting the extracellular matrix molecules, such as collagen and elastin, giving the support and elasticity to the skin and mucosa (Gawkrodger, 2002; Morasso and Tomic-Canic, 2005). Moreover, the connective tissue of the skin and mucosa supports the vascular network that is critical for the survival of both epithelium (which has no blood vessels) and connective tissue. Unlike mucosa, skin contains appendages such as hair follicles and sweat glands that are originated from the epidermis but penetrate deeply into the dermis (Gawkrodger, 2002). Any injury to the integrity of the skin and mucosa normally initiates the process of wound healing as the body's pivotal survival response, which includes coverage of the wound through re-epithelialization and restoration of the damaged connective tissue (Clark, 1996).

1.2. Wound healing

1.2.1. Overview

Wound healing is composed of accurately controlled processes in the epithelium and connective tissue that restore tissue integrity after injury. It involves coordinated function and interaction of epithelial cells, inflammatory cells, and connective tissue cells. These cell-to-cell interactions are mediated mainly by the interplay of growth factors and cytokines, cell surface receptors (more specifically integrins), and the specific molecules in extracellular matrix (ECM) (Giancotti and Ruoslahti, 1999; Li et al., 2007; Eckes et al., 2010). The misregulation of wound healing events results in rather devastating consequences ranging from chronic non-healing wounds to different types of scars (Clark, 1996; Ferguson and O'Kane, 2004).

1.2.2. Stages

Upon injury, a series of overlapping events takes place in the wounded tissue (most extensively studied in cutaneous wound healing) which includes early wound healing events (hemostasis and inflammation), proliferative phase (re-epithelialization and granulation tissue formation), and maturation and tissue remodeling (Li et al, 2007) (Figure 1.1). Although the focus of this dissertation is more on the epithelial cells and therefore epithelial wound healing (re-epithelialization), one should always consider the impact of different stages of wound healing on one another and the interplay of epithelial cells and other cell lines (mediated by growth factors) that would determine the final wound outcome. Here, therefore, I have reviewed all the stages of wound healing with a special attention to the role of growth factors as messengers of cellular interactions.

1.2.2.1. Early wound healing events (hemostasis and inflammation)

Upon wounding, contact of circulating platelets to damaged tissue and exposed collagen results in platelets aggregation and formation of a hemostatic plug (Diegelmann and Evans, 2004). Aggregating platelets release clotting factors resulting in the deposition of a blood clot that is mostly composed of fibrin and fibronectin (Diegelmann and Evans, 2004). This blood clot provides the first barrier against bacteria, prevents further loss of blood or plasma, entraps proteins and platelets, and serves as a provisional matrix (Diegelmann and Evans, 2004;

Midwood et al., 2004; Schäfer and Werner, 2007). The wound provisional matrix is a substrate that facilitates cell migration into wound both from the circulation and from the wound edges (Schäfer and Werner, 2007). Meanwhile, the release of soluble mediators from degranulating platelets and the serum of injured blood vessels stimulates the inflammatory response and cell migration into the wound provisional matrix (Schäfer and Werner, 2007).

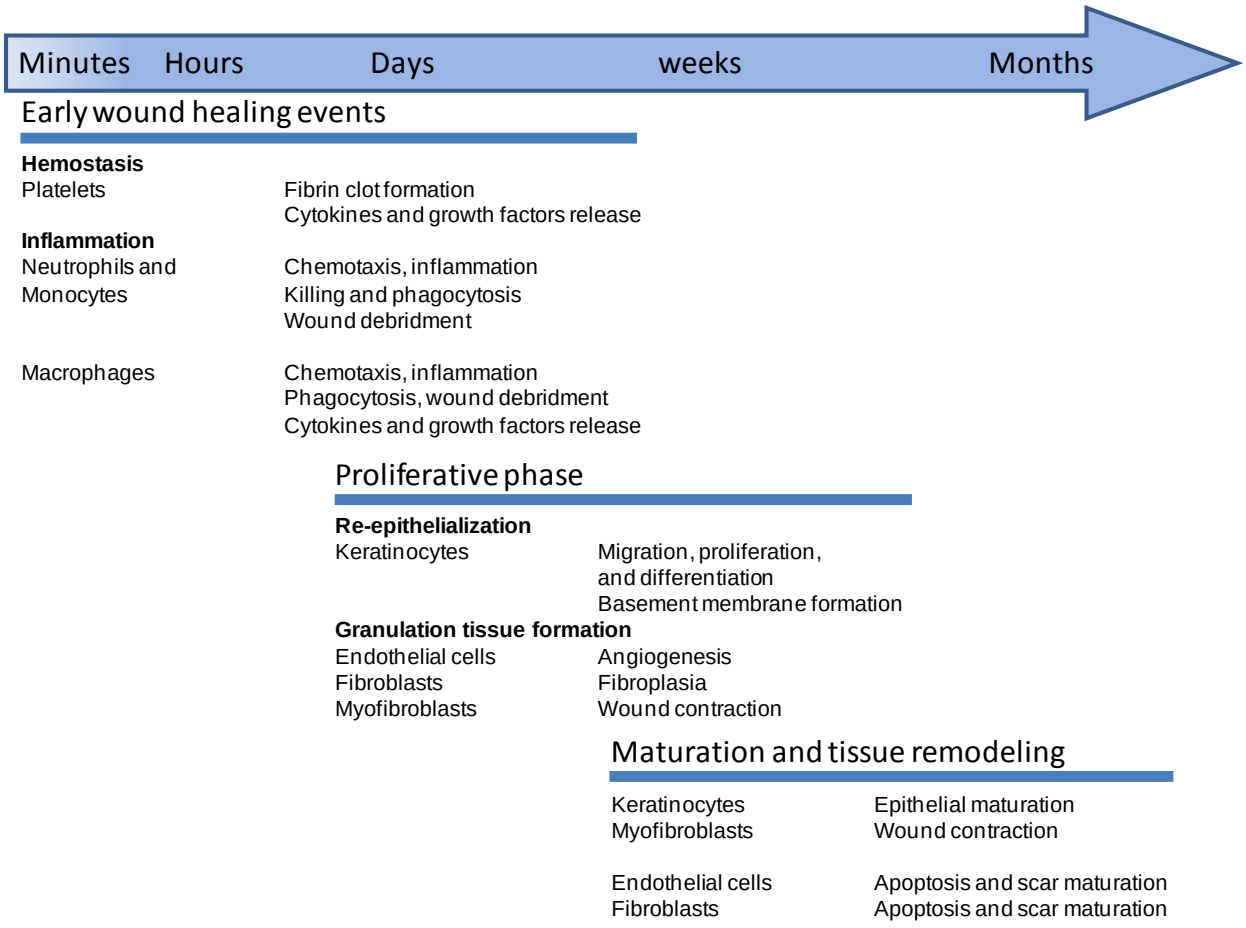

Figure 1.1. Wound healing events (modified from Li et al., 2007).

Within minutes after injury, neutrophils invade the wound and remain the prominent inflammatory cell type detected in the wound site for the first 24 hours after injury. Integrin receptors expressed on the surface of neutrophils enhance their interactions with the molecules of the provisional matrix. Such interactions allow neutrophils to effectively remove foreign materials, bacteria and cell debris from the wound bed (Simpson and Ross, 1972; Diegelmann and Evans, 2004; Li et al., 2007). Neutrophils are also a good source of pro-inflammatory cytokines released to the wound (Hübner et al., 1996). The number of neutrophils declines at the wound site within few days in the absence of infection (Hantash et al., 2008). Neutrophil infiltration to the wound is followed by circulating monocytes residing in the wound and converting to large phagocytic macrophages about two days after injury, the process that is stimulated by the transforming growth factor β (TGF-β) (Diegelmann and Evans, 2004; Li et al., 2007). These specialized wound macrophages are considered as the most important inflammatory cells involved in normal wound healing (Diegelmann and Evans, 2004). Wound macrophages are not only responsible for the phogocytosis of the debris from dead cells, bacteria-filled neutrophils, and other micro-intruders to the wound, but also they provide the wound with the continuous supply of the essential cytokines and growth factors, such as platelet-derived growth factor (PDGF) and TGF-β, which are required for the transition between inflammatory response and proliferative phase (Diegelmann and Evans, 2004; Li et al., 2007).

1.2.2.2. Re-epithelialization

Re-epithelialization is an essential part of wound healing which restores the intact epidermal barrier through directed migration, proliferation, and differentiation of keratinocytes. Defects in the process of re-epithelialization could result in clinical condition of chronic non-healing wounds (Sivamani et al., 2007). Re-epithelialization begins within hours after wounding (Hantash et al., 2008). Basal and a subset of suprabasal keratinocytes from wound edges or from the neighboring hair follicle bulge or other skin appendages in case of cutaneous wounds are the first who migrate into the wound provisional matrix (Larjava et al., 2002; Li et al., 2007; Hantash et al., 2008). Bone marrow-derived stem cells which enter the circulation also home to the wound site and differentiate into keratinocytes to assist wound re-epithelialization (Badiavas et al., 2003; Wu et al., 2010). Migratory keratinocytes go through several phenotypic changes that enable them to move. These changes include dissolution of cell-to-cell and cell-to-basement

membrane contacts (desmosomes and hemidesmosomes, respectively) (Hantash et al., 2008). Moreover, polarized basal keratinocytes become elongated and flattened (Larjava et al., 2002). These phenotypic changes are associated with some molecular events not present in normal circumstances which include the upregulation or neo-expression of certain integrins (fibronectin/tenascin- and vitronectin-binding integrins), and the re-distribution of the others (collagen/laminin-binding integrins) so that the epidermal sheet can attach to and move forward over the components of the wound provisional matrix (Clark, 2001; Larjava et al., 2002; Martin and Parkhurst, 2004; Hantash et al., 2008). Migrating keratinocytes are highly phagocytic. They produce proteinases such as plasminogen activator, as well as MMPs (e.g., MMP-1, MMP-9, and MMP-10), and therefore could dissect between collagenous dermis and fibrin eschar in cutaneous wounds, or break through the provisional matrix in case of oral mucosal wounds (Larjava et al., 2002; Hantash et al., 2008). The phenotypical changes observed in the keratinocytes at the edges of cutaneous wounds resemble certain aspects of epithelial-to-mesenchymal transition (EMT), the process that converts epithelial cells into motile mesenchymal cells (Arnoux et al., 2005). The EMT is a critical step during embryonic development and tumor metastasis (Arnoux et al., 2005; Savagner et al., 2005). It involves several events: a) loss of desmosomes and adherens junctions that are important in intercellular adhesion; b) loss of cell polarity; c) increased secretion of ECM-degrading proteinases; d) cytoskeletal changes which include a shift from keratin to vimentin intermediate filaments; and e) cell motility (Arnoux et al., 2005). Migratory wound keratinocytes have shown partial detachment of intercellular adhesion. They also expressed vimentin and fibroblast-specific protein-1 (FSP-1) which are among characteristics of mesenchymal cells (Yan et al., 2010). Activation of TGF-β pathways has been shown to be the key regulator of EMT during development and in late-stage carcinomas (Arnoux et al., 2005). TGF-β is also present at the wound site from the very early stages of wound healing, and along with other inflammatory cytokines (*e.g.*, tumor necrosis factor-α or TNF-α) is implied in the induction and regulation of EMT (Yan et al., 2010). It is noteworthy that unlike tumor metastasis, EMT as a part of re-epithelialization is completely reversible (Arnoux et al., 2005).

About 48 to 72 hours after wounding, keratinocytes that are located near the migrating tongue start to proliferate. This proliferating source of keratinocytes is to provide enough supply

of cells for wound coverage (Li et al., 2007). The exact signals initiating and regulating re-epithelialization are not totally understood. The lack of contact inhibition due to the wound defect may induce the migratory phenotype in keratinocytes (Singer and Clark, 1999). More importantly, growth factors and cytokines such as epidermal growth factor (EGF) family members, TGF-βs, and fibroblast growth factors (FGFs) play critical roles in stimulating epithelial cell migration, proliferation, and differentiation (Barrientos et al., 2008). It is noteworthy that the nature of the extracellular matrix (ECM) underneath the keratinocytes is an important determinant of how keratinocytes respond to growth factors, since the composition of ECM not only greatly differs in normal tissue when compared to the wounded tissue, but also varies in early and late stages of wound healing (Kirfel and Herzog, 2004; Sivamani et al., 2007). As soon as wound is fully covered, keratinocytes undergo stratification and re-differentiation in order to re-establish the barrier function (Clark, 1996; Schäfer and Werner, 2007). As re-epithelialization proceeds, keratinocytes deposit the proteins required for the reformation of the basement membrane zone (BMZ), which was damaged due to injury, in a very orderly fashion from the margin of the wound to the centre. Reformation of BMZ occurs within 7 to 9 days after re-epithelialization (Clark, 2001; Li et al., 2007). Concurrently, epithelial cells regain their normal phenotype, polarity and firm attachments to each other and to the newly reformed basement membrane (Clark, 2001). Re-epithelialization must be coordinated with the migration and proliferation of fibroblasts and the formation of new blood vessels in the underlying wound bed, called the granulation tissue (Clark, 2003). In this context, keratinocytes influence fibroblasts and endothelial cells directly or indirectly through the secretion of the growth factors such as platelet-derived growth factor (PDGF) which is a major mitogen and motogen of fibroblasts and vascular endothelial cell growth factor (VEGF) which potentiates angiogenesis (Brown et al., 1992; Ansel et al., 1993; Heldin and Westermark, 1996; Clark, 2003). Moreover, keratinocytes produce cytokines that both activate epithelial and mesenchymal cells (*e.g.*, HB-EGF and TGF-β isoforms) (Clark, 2003).

1.2.2.3. Granulation tissue formation

About 4 days after injury, the granulation tissue begins to evolve (Hantash et al., 2008). The number of fibroblasts and macrophages increases at the wound site and numerous new capillaries develop, giving the new matrix its granular appearance (Schäfer and Werner, 2007).

Early wound provisional matrix serves as a scaffold for activated macrophages which provide a continuous source of growth factors required for fibroplasia (*i.e*, accumulation of fibroblasts and production of new collagen and other ECM molecules) and neovascularization (Li et al, 2007, Hantash et al., 2008). Moreover, growth factors, especially PDGF and TGF-β1, stimulate fibroblasts to migrate into the wound site from different sources to deposit a collagen-rich matrix composing of collagens, proteoglycans, and elastin (Singer and Clark, 1999; Li et al., 2007). The main source of migrating fibroblasts is the surrounding connective tissue, where the cells start to proliferate as a response to injury and migrate into provisional matrix around day 4 post-wounding (Häkkinen et al., 2000a; Li et al, 2007). Furthermore, perivascular cells (pericytes) surrounding blood vessels and fibrocytes (which are bone marrow-derived mesenchymal progenitors that enter the circulation) have been found to be other sources of wound fibroblast population (Abe et al., 2001; Mori et al., 2005; Rajkumar et al, 2006; Bellini and Mattoli, 2007; Fan and Liang, 2010). Fibroblasts at the wound site are mainly characterized by their TGF-β-mediated ability in the synthesis, deposition, and remodeling of the collagen-rich extracellular matrix. This newly formed collagen-rich extracellular matrix gradually replaces the early wound provisional matrix (Roberts and Sporn, 1996; Li et al., 2007). However, depending on their tissue sources, wound fibroblasts can also have other functions, including the regulation of the inflammatory reaction and wound contraction (Häkkinen et al., 2000a). Moreover, fibroblasts have the capacity to secrete different cytokines that could stimulate themselves through autocrine or paracrine ways, or selectively induce epithelial cells in a paracrine manner (Clark, 2003). The keratinocyte growth factor-1 and -2 (which are the members of FGF family) and the insulin-like growth factor-1 (IGF-1) are good examples of fibroblast-derived cytokines that stimulate keratinocytes (Tavakkol et al., 1992; Igarashi et al., 1998; Putnins et al., 1999). During the second week post-wounding some of the wound fibroblasts transform into myofibroblasts, the cells that are characterized by the abundance of cytoplasmic actin microfilaments along their plasma membrane and increased expression of α-smooth muscle actin (Darby et al., 1990; Li et al, 2007). The appearance of myofibroblasts in the wound is induced by TGF-β and coincides with wound contraction and completion of re-epithelialization, and hence wound closure (Desmoulie`re, 1993).

Blood vessels carry oxygen and nutrients to the cells and, therefore, angiogenesis is a critical process that sustains the granulation tissue formation. Angiogenesis refers to the formation of new blood vessels by the migration of endothelial cells from capillaries adjacent to the wound and sprouting into wound provisional matrix (Li et al, 2007). Like keratinocytes and fibroblasts, endothelial cells also undergo phenotypical changes including formation of cytoplasmic pseudopodia on day 2 post-wounding that enable them to migrate into the wound (Kalebic et al., 1983; Li et al., 2007). The wound provisional matrix and its gradual replacement with the new ECM deposited by fibroblasts support endothelial cells residing in the wound and neovascularization (Feng et al., 1999; Li et al., 2007). A decreased level of oxygen in the damaged tissue is the first factor that stimulates angiogenesis. The hypoxia-induced release of VEGF from epithelial cells as well as macrophage-released bFGF (basic fibroblast growth factor) and TGF-β are known to be strong inducers of the endothelial cell migration and angiogenesis (Li et al, 2007). During angiogenesis, endothelial cells, in turn, secrete cytokines necessary for the healing process (Li et al., 2007).

As soon as collagen deposition reaches a critical level, fibroblast proliferation and matrix synthesis are slowed down and angiogenesis stops (Grinnel, 1994, Hantash et al., 2008). Subsequently, many fibroblasts, as well as many new blood vessels are removed from the matrix through apoptosis (Desmoulière et al., 1995; Ilan et al., 1998; Hantash et al., 2008). Collagen contraction has been shown to induce apoptosis in fibroblasts during wound healing. Higher activity of cross-linking tissue transglutaminase in fibroblasts derived from hypertrophic scars has been associated with the resistance of those fibroblasts to collagen-contraction-induced apoptosis (Linge et al., 2005). Expression of nitric oxide synthase induced by different cytokines, production of reactive oxygen species, expression of PPARs (peroxisome-proliferator-activated receptors), and expression of cytokines such as interferon γ and α2b are among other factors implicated in regulation of apoptosis during wound helaing (Nedelec et al., 1998; Akasaka et al., 2000; Weller, 2003; Tan et al., 2004) .

1.2.2.4. Tissue remodeling and maturation

The maturation of collagen fibers and remodeling of extracellular matrix are the last phases of wound healing. At this stage, the type III collagen that is abundant in the granulation tissue is gradually degraded and replaced with mature type I collagen bundles, which are

synthesized by fibroblasts and become organized along the lines of tension at the wound site (Risteli et al., 1993; Li et al., 2007; Hantash et al., 2008). Moreover, the number of fibroblasts and blood vessels progressively declines (Hantash et al., 2008). The old matrix components of the granulation tissue are removed through phagocytosis by fibroblasts and macrophages and by the activity of their secreted MMPs. MMPs are found at very low levels in healthy normal tissues, but are highly induced in response to cytokines during wound healing. The activity of MMPs is also controlled by tissue inhibitors of metalloproteinases. The balance between the activity of MMPs and their inhibitors is a key factor for normal remodeling of wounds (Steffensen et al., 2001; Visse et al., 2003). The normal result of maturation and remodeling step is the formation of a scar, a hypo-cellular and pale tissue which in case of skin is without skin appendages (e.g., hair follicles and sebaceous glands) (Hantash et al., 2008). The newly formed dermis or the connective tissue of the scar normally continues remodeling for months or years (depending on the size and location of the wound) to gain more and more of its pre-injury strength (Li et al, 2007).

1.2.3. Scars

The disruption of collagen remodeling by a number of factors can lead to the formation of raised fibroproliferative scars in the skin (Ferguson and O'Kane, 2004; Hantash et al., 2008). These scars can range from clinically insignificant cases to more severe cases of hypertrophic scars and keloids with devastating cosmetic and debilitating consequences (Armour et al., 2007). Fibroproliferative scars result from an imbalance between the ECM synthesis and degradation, leading to excessive connective tissue formation (Hantash et al., 2008). This imbalance could begin at very early stages of wound healing with increased secretion or activity of pro-fibrotic growth factors such as TGF-β1 (O'Kane and Ferguson, 1997). Increased expression of PDGF by platelets and macrophages, increased expression of connective tissue growth factor (CTGF) by fibroblasts, and higher levels of interleukin-4 (IL-4) and IL-13 produced by T lymphocytes (T helper-2 cells) as a result of an excessive inflammatory response are also involved in over-production of the ECM and formation of fibroproliferative scars (Igarashi et al., 1996; Niessen et al., 2001; Colwell et al., 2005; van der Veer et al., 2009). Furthermore, disturbances in the keratinocyte-fibroblast cross-talk through cytokine network, and reduced collagenolysis could result in impaired tissue remodeling and scarring (Ghahary et al., 1996; Rahban and Garner,

2003; Ghahary and Ghaffari, 2007). Despite our present knowledge about the underlying mechanisms of scarring, there is no predictable method for prevention or therapy of scars (Armour et al., 2007).

An extremely important observation in this regard is the fact that healing in the fetal skin, as well as in the adult oral mucosa, leaves no scar (Häkkinen et al., 2000a; Ferguson and O'Kane, 2004; Mak et al., 2009; Wong et al., 2009). This observation could potentially be the key to find answers to the question as to why wounds in the adult skin produce scar. Much research has been conducted to unveil the underlying mechanisms of fetal versus adult cutaneous wound healing. Surprisingly, however, only few studies have been performed on wound healing in the more accessible tissue of the oral mucosa versus skin. Therefore, for better understanding of the underlying mechanisms of scarless healing, in the following sections, scarless wound models will be compared with scar-forming wound models.

1.2.4. Scarless fetal versus scar-forming adult wound healing

For years, it has been known that fetal wounds in mammalian species heal without scarring (Whitby and Ferguson, 1991; Ferguson and O'Kane, 2004). Scar-free healing in fetal wounds is determined by the gestational age and size of the wound and it is also organ-dependent (*i.e.*, not all embryonic organs are able to completely regenerate) (Lorenz and Adzick, 1993; Cass et al., 1997). It has been shown that cutaneous fetal wounds heal scarlessly prior to 24-weeks of gestation in humans (Lorenz et al., 1992). Furthermore, wounds with a larger size heal without scarring only at earlier gestational ages. This suggests that the ability of healing without scar diminishes as the gestational age progresses (Cass et al., 1997). Although scarless repair is inherent to the developing skin of the fetus and is also age-dependent, it has been found that wound manipulations could reduce scarring in adult cutaneous wounds or induce scar formation in scarless fetal wounds (Ferguson and O'Kane, 2004; Hantash et al., 2008). For example, the application of neutralizing antibodies to TGF-β1 or TGF-β2 (or both) resulted in significantly reduced scarring in adult rodent wounds (Shah et al., 1992, 1994; 1995). On the other hand, the exogenous application of TGF-β3 considerably reduced scarring in healing adult wounds (Shah et al., 1995). Moreover, mouse embryos which were homozygous null for TGF-β3, unlike their wild-type counterparts, showed delayed wound healing and developed scars (Proetzel et al.,

1995). The underlying mechanisms of fetal scarless wound healing are still under investigation. However, the relative lack of inflammation (McCallion and Ferguson, 1996), faster re-epithelialization (Whitby et al., 1991; Cass et al., 1998), special characteristics of fetal fibroblasts and ECM composition (Hantash et al., 2008), a higher rate of angiogenesis (Yang et al, 2003), and the pattern of cytokine expression in fetal wounds (which is different from that of adult wounds) could in part explain the scarless phenotype observed in fetal wound healing (Hantash et al., 2008).

The relative lack of inflammation in scarless fetal wounds has been associated with the decreased platelet degranulation and aggregation and the lower production of cytokines including PDGF, TGF-β1 and TGF-β2 by those cells (Olutoye et al., 1996). Moreover, there is a lower number of neutrophils present at the fetal wound site with less ability of phagocytosis in comparison to high numbers of vigorously phagocytic neutrophils in adult wounds (Jennings et al., 1991; Li et al., 2007). It has been shown that pro-inflammatory cytokines, such as IL-6 and IL-8, stay at least for a few days in adult wounds, but disappear within hours from fetal wounds. Adding IL-6 to scarless fetal wounds has been shown to result in scarring (Hantash et al., 2008).

Rapid re-epithelialization results in faster healing and less scar formation (Whitby et al., 1991; Cass et al., 1998). Scarless fetal wounds have shown accelerated re-epithelialization when compared with adult wounds (Cass et al, 1996, 1998). The underlying mechanisms for this rapid coverage of fetal wound by keratinocytes remains unclear, although a number of reasons have been suggested. It has been shown that the upregulation and neo-expression of epidermal integrins occur faster in fetal wound keratinocytes than their adult counterparts. This could potentially facilitate cell adhesion and migration and promote accelerated re-epithelialization in scarless fetal wounds (Cass et al., 1998; Kirfel et al., 2003). Another piece of evidence for rapid migration of keratinocytes into cutaneous wounds is the formation of the actin cables within just two minutes of wounding in the epithelial cells at wound edges in both chick and mouse embryos. This has not been the case in adult wounds (Martin and Parkhurst, 2004). Moreover, it has been shown that fetal keratinocytes may modulate the expression of key cytokines differently than those from adults. Recent *in vitro* studies have shown that fetal keratinocytes modulate TGF-β expression and its signaling mediators such as smads and display an overall antifibrotic effect on fetal fibroblasts in co-culture conditions (Colwell et al., 2007a, 2007b).

Fetal fibroblasts show specific characteristics that may contribute to the scarless phenotype of fetal wounds. It has been shown that fetal fibroblasts have higher migratory activity on type I collagen and hyaluronic acid (HA) gel than adult fibroblasts (Park et al., 2001). Fetal skin contains a higher level of HA in the ECM and a higher number of HA receptors expressed by fetal fibroblasts when compared with adult skin fibroblasts (Mast et al., 1991, 1993). These factors could potentially facilitate a faster migration of fetal fibroblasts into the wound site when compared with adult fibroblasts (Park et al., 1991). It has been shown that the initiation of scar formation in developing fetal wounds is concurrent with the induction of fibroblast into myofibroblast differentiation (Estes et al., 1994). In addition, myofibroblasts remain present longer in scar forming wounds as compared to scarless wounds (Darby and Hewitson, 2007).

Collagens are the main components of both adult and fetal ECM, and are mainly produced by fibroblasts. The optimal production, deposition and organization of collagens, therefore, are critical factors in the process of normal wound healing with no or minimal scarring (Hantash et al., 2008). There are rather important differences in collagen deposition and organization during scarless versus scar-forming wounds. It has been shown that fetal fibroblasts produce different types of collagen in higher levels than adult fibroblasts (Gosiewska et al., 2001; Brink et al., 2005). Furthermore, fetal fibroblasts start producing collagen right after injury, whereas collagen synthesis is delayed by adult fibroblasts (Hantash et al., 2008). Unlike scar tissues, which show an abnormal organization of collagen fibers, fetal cutaneous wounds heal with a fine reticular and basket-weave appearing ECM which is identical to that of normal skin (Longaker et al., 1990, 1994). It is noteworthy that although type I collagen is the most abundant collagen present in scar tissue, fetal skin contains a higher ratio of type III collagen to type I collagen (Merkel et al., 1988). The expression of lysyl oxidase (an enzyme that cross-links collagen and elastin) has been shown to be higher in later gestational ages when wounds begin to heal with scarring. This may suggest a role for lysyl oxidase in promoting an abnormally organized ECM and scar formation (Colwell et al., 2006). MMPs and tissue inhibitors of MMPs (TIMPs) are essential factors in maintaining the balance of collagen synthesis and degradation. This balance is in turn critical in the healing process. It has been shown that in mid-gestation fetal dermal wounds, MMPs are expressed in levels higher than in adult wounds, suggesting an important role for MMPs in scarless healing (Bullard et al., 1997).

There have been contradictory reports regarding the regulation of proteoglycans with anti-fibrotic properties in the ECM during early fetal wound healing. For example, the expression of fibromodulin has been shown to be increased significantly in scarless fetal wounds, while the expression of decorin, another anti-fibrotic proteoglycan, was suppressed (Soo et al., 2000; Beanes et al., 2001a). This is surprising since decorin has been well-characterized for its anti-fibrotic and anti-scarring effects in adult wound healing (Scott et al., 1995; Grisanti et al., 2005). Further studies are required to clarify the role of proteoglycans in regards to scarless versus scar-forming wound healing.

Interestingly fetal and adult fibroblasts respond to TGF-β differently. That is, TGF-β inhibits the proliferation of fetal fibroblasts while it induces the proliferation of fibroblasts in adulthood (Giannouli and Kletsas, 2006). It has been found that some growth factors – such as TGF-βs – with pivotal roles in many steps of wound healing show a different pattern of the expression in fetal versus adult wounds in several stages of wound healing (Ferguson and O'Kane, 2004; Hantash et al., 2008). In rodent models there was an upregulation in the expression of TGF-β1 and -β2 in adult skin wounds, while these TGF-β isoforms remained unchanged in fetal wounds (Krummel et al., 1988; Nath et al., 1994). In addition, an exogenous addition of fibrogenic TGF-β1 to scarless fetal wounds has been associated with scar formation, while the treatment of adult wounds with neutralizing antibody against TGF-β1 and TGF-β2 reduced scar formation (Nath et al., 1994; Shah et al., 1994). *In vitro* studies have shown that the expression of TGF-β1, TGF-β2, TGF-β receptor I and II, and the mediators of TGF-β signaling (Smad2, Smad3, and Smad4) are lower in fetal fibroblasts than post-natal fibroblasts (Colwell et al., 2007a). Unlike many studies that have shown lower expression levels of TGF-β1 in fetal wounds, Goldberg et al. (2007) found an increased TGF-β1 expression in mid-gestational wounds of a murine model. Considering contradictory reports regarding the expression levels of TGF-β isoforms in different animal models, the general view is that the relative ratios of TGF-β isoforms may be a more important determinant of the wound final phenotype than their absolute levels of protein expression (Li et al., 2006; Hantash et al., 2008). In scarless fetal wounds, the expression level of TGF-β3 is increased while the expression level of TGF-β1 does not change. Scar forming wounds, however, have been associated with an increased expression level of TGF-β1 and decreased expression level of TGF-β3. Interestingly, the application of TGF-β3, also

called anti-fibrogenic isoform of TGF-β, on adult incisional wounds leads to reduced scar formation (Ferguson and O'Kane, 2004). Fetal fibroblasts have been found to have increased expression level of TGF-β3 (Colwell et al., 2007b; Goldberg et al., 2007). Furthermore, it has been shown that the expression of an important marker of fibrosis and downstream molecule of TGF-β, plasminogen activator inhibitor-1, was upregulated in an *ex vivo* fetal limb wound of TGF-β3 knockout mice (Li et al., 2006). Taken together, these data are suggesting a key role for relative ratios of TGF-β1 (and TGF-β2) to TGF-β3 in scar formation with TGF-β1 predominating in scars and TGF-β3 associating with less scarring and better healing.

It has been shown that pro-fibrotic cytokines PDGF and FGF have a prolonged expression in adult wounds, while they disappear from fetal wounds very quickly. The exogenous treatment of scarless fetal wounds with PDGF results in a significant inflammatory response and a higher activity of fibroblasts, two important factors that promote excessive collagen deposition and scar formation (Whitby and Ferguson, 1991; Haynes et al., 1994).

The expression of VEGF, which is a mitogen for endothelial cells, however, has been shown to significantly upregulate in scarless wounds while its expression remains unchanged in fetal wounds that form scars. Higher stimulus for angiogenesis, therefore, may be important in faster healing of fetal wounds (Yang et al., 2003; Beanes et al., 2001b).

Aside from the inherent characteristics of fetal versus adult cells that are central in determining the wound outcomes, there are other important factors, such as the expression level and pattern of different cytokines, that contribute in scarless versus scar-forming wound phenotype. A better identification of those factors would allow us to plan for therapeutic manipulations of adult scar-forming wounds. Manipulations of TGF-β isoforms is a good example of such approach in reducing scar formation in adult cutaneous wounds, as discussed earlier.

1.2.5. Skin versus gingival wound healing

Both mucosal and cutaneous wounds share similar steps of healing as discussed earlier. Interestingly, however, adult oral mucosal wounds show accelerated healing with no or minimal scarring compared to adult skin wounds (Häkkinen et al. 2000a; Szpaderska et al. 2003; Mak et

al., 2009; Wong et al, 2009). The mechanisms underlying scarless oral wound healing have not been fully studied. The absence of scar in oral mucosal wounds may in part be due to the growth factors, such as EGF, VEGF, bFGF, TGF-β, and IGF (Skaleric et al., 1997; Taichman et al., 1998; Häkkinen et al., 2000a; Fujisawa et al., 2003), and MMPs present in the saliva, specific microflora in the oral cavity that, in turn, would produce different cytokines or attract cytokine-producing cells, the fetal-like phenotype of the cells and the specific composition of ECM in the oral mucosa (Sciubba et al. 1978; Schor et al. 1996; Häkkinen et al. 2000a; Szpaderska et al. 2003; Schrementi et al. 2008).

Oral mucosal wounds are associated with a reduced inflammatory response, the condition that mimics the lack of inflammation in scarless fetal wounds (Sciubba et al., 1978; McCallion and Ferguson, 1996; Szpaderska et al., 2003; Mak et al., 2009). Early wounds in mouse oral mucosa contain a lower number of inflammatory cells (including neutrophils, macrophages, and T cells) and lower level of pro-inflammatory cytokines (IL-6 and IL-8) compared to cutaneous wounds (Szpaderska et al., 2003). Soon after wounding, a lower expression level of pro-fibrotic TGF-β1 and a relatively higher level of anti-fibrotic TGF-β3 were found in oral mucosal wounds, but not in cutaneous wounds (Szpaderska et al, 2003; Schrementi et al., 2008). Similarly, in scarless fetal wounds the ratio of TGF-β3 expression to TGF-β1 has been significantly higher than in scar-forming adult wounds (Ferguson and O'Kane, 2004).

Similar to the explanation as to why fetal wound healing is scar-free, a main reason for a scarless phenotype observed in oral mucosal wounds with reduced scar formation is the inherent characteristics of the tissue itself. Fetal-like fibroblasts with their unique response to wounding and specialized epithelium and connective tissue of oral mucosa are considered as such intrinsic factors (Häkkinen et al., 2000a). It has been shown that adult gingival fibroblasts share many similarities with fetal fibroblasts in their growth, migration, phenotype, and production of and responsiveness to growth factors (Schor et al., 1996). For instance, oral mucosal fibroblasts migrate and populate the wound site more rapidly than dermal fibroblasts (al-Khateeb et al., 1997). Also, unlike dermal fibroblasts, gingival fibroblasts have the capacity to re-organize and degrade fibrin clot and ECM rapidly. This capacity is in part due to high expression levels of tissue plasminogen activator and secretion of proteolytic enzymes, including MMP-2, MMP-3, and MMP-13 (Stephens et al., 1996; Lorimier et al., 1996, 1998; Westermarck and Kähäri, 1999;

Sukotjo et al., 2003; Shannon et al., 2006). It has been shown that gingival wounds contain a lower number of cells expressing type I procollagen – the main component of scar tissue – and a lower content of fibronectin, when compared with cutaneous scars (Wong et al, 2009). Moreover, there are differences between the types and amounts of some glycosaminoglycans and proteoglycans secreted by oral mucosal versus dermal fibroblasts (Larjava et al., 1988). Other intrinsic factors of oral mucosa have been also implied as possible facilitators of reduced scarring in oral mucosal wound healing; factors such as a high turnover rate of connective tissue and epithelium, and a high number of blood vessels (Häkkinen et al., 2000a).

Cross-talks among growth factors, integrins and the ECM play crucial roles in controlling cellular functions during dermal repair and scarring (Eckes et al., 2010). A good example of such interplay is the activation of TGF-β by αvβ6 integrin, which is highly expressed by keratinocytes during conditions such as wound healing. The αvβ6 integrin binding to the latent TGF-β incorporated to the ECM has been shown to result in mechanical forces which induce conformational changes in the latent molecule of TGF-β and its activation (Breuss et al., 1995; Annes et al., 2004). The functionality of such mechanism of activation should be further investigated during *in vivo* conditions. Our knowledge to date about αvβ6 integrin-mediated activation of TGF-β will be discussed later in this chapter, but first a general overview on "integrins" will be given. Next, our present knowledge about the αvβ6 integrin will be discussed followed by an introduction to the TGF-β in general and during wound healing.

1.3. Integrins

1.3.1. Overview and function

Integrins form a large family of cell adhesion molecules that are mainly involved in cell-to-cell and cell-to-extracellular matrix interactions (Hynes, 2002). They connect extracellular and intracellular spaces through binding to ligands outside the cell and cytoskeletal and signaling molecules inside the cell, thereby transmitting signals through plasma membrane bidirectionally. They can also respond to growth factors and cytokines through intracellular signaling (Takada et al., 2007). Those signals determine the subsequent cell responses (e.g., migration, differentiation, motility, and survival) (Evans and Calderwood, 2007; Harburger and Calderwood, 2009). Integrins, therefore, play critical roles in many normal cellular and pathological conditions. They

have several key roles in development, tissue repair, homeostasis, immune response, and cancer (Hynes, 2002; Luo and Springer, 2006; Gahmberg et al., 2009). Data from integrin knockout mice models have revealed both redundant and non-redundant functions of integrins (Gahmberg et al., 2009). Namely, some integrins could bind to similar ligands and thus substitute for one another if required (redundant function) while other integrins are unique in their ligand binding and function (non-redundant). A deficiency in some integrins (especially with non-redundant functions) results in serious consequences such as perinatal lethality and developmental defects (Hynes, 2002). The interaction of integrins with their ligands is a major area of research in investigating the mechanisms of many pathological conditions and finding good targets for the development of therapeutic drugs (Haynes, 2002).

1.3.2. Ligands

Integrins have been shown to interact with different ECM proteins, cell surface ligands, transmembrane proteins, soluble proteases, pathogens and growth factors (van der Flier and Sonnenberg, 2001). Mammalian integrins can be classified based on their ligand specificity as follows: laminin-binding integrins, collagen-binding integrins, leukocyte integrins, and RGD recognizing integrins (e.g., fibronectin-binding integrins) (Figure 1.2) (Takada et al., 2007). Integrins can be further defined based on the presence or absence of a motif called I-domain in the extracellular part of their α chain (*i.e.*, I-domain and non-I-domain integrins) (Figure 1.2) (Takada et al., 2007). It is noteworthy that some integrins have high specificity for only one ligand, while many other integrins can recognize and bind to multiple ligands (Gahmberg et al., 2009).

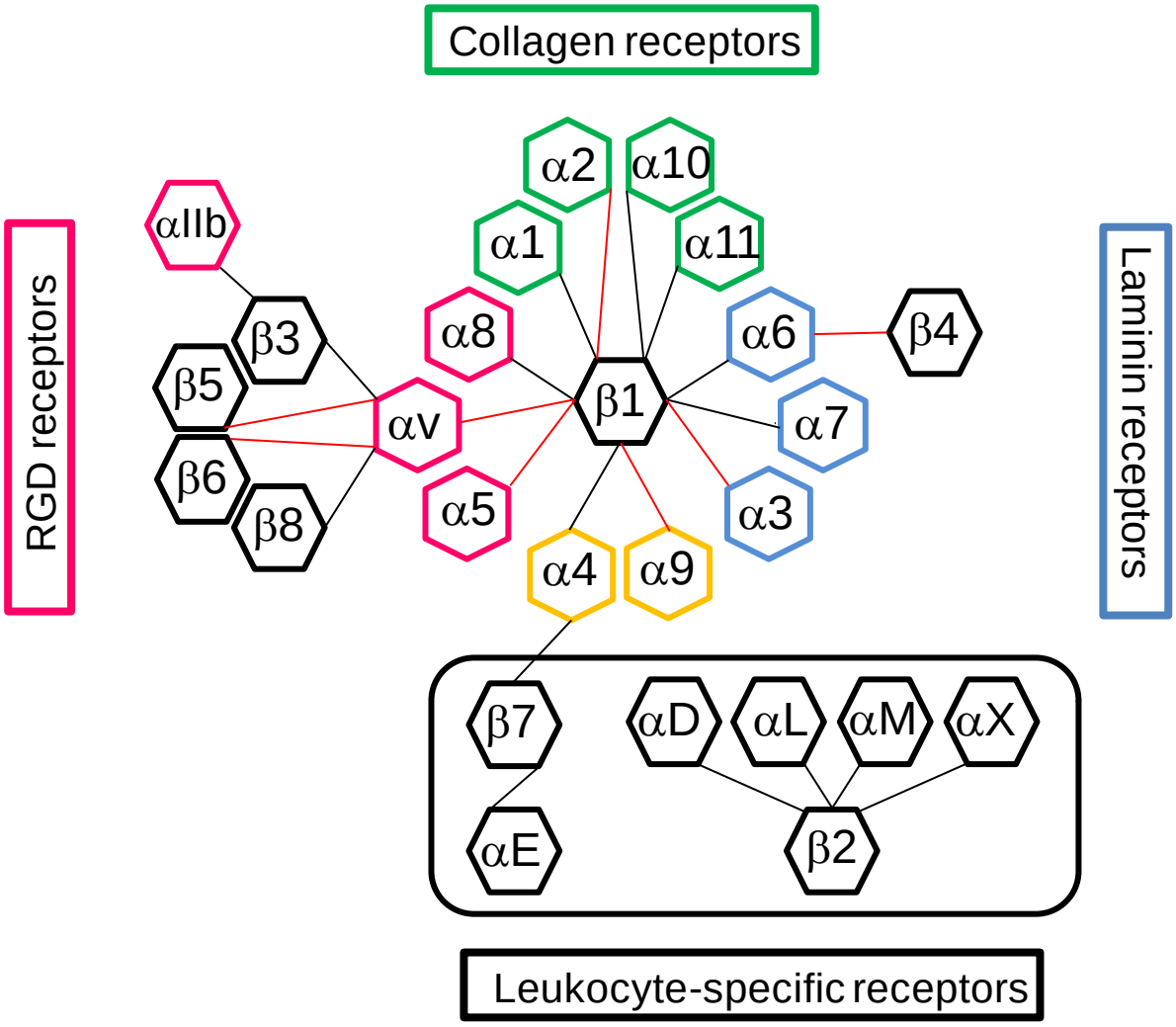

Figure 1.2. The integrin superfamily. The integrin subunits that are related with a "red line" are expressed in the basal keratinocyte of normal or wounded epithelium (modified from Thomas et al., 2006).

1.3.3. Integrin structure

Integrins are composed of two distinct non-covalently bound transmembrane glycoproteins called α and β subunits (Calderwood, 2004; Gahmberg et al., 2009). There are at least 18 alpha and 8 beta subunits which can diversely combine to make more than 24 different integrin heterodimers in humans (Gahmberg et al., 2009) (Figure 1.2). Each of α and β subunits contains large extracellular domains, a transmembrane domain, and a short cytoplasmic domain

(Gahmberg et al., 2009). The extracellular part of the integrins consists of multiple domains which form a ligand binding head and two legs (Gahmberg et al., 2009). There is a so-called I-domain in about half of α subunits (*e.g.*, collagen-binding integrins) (Hynes, 2002). When the I-domain is present in the α subunit, it solely serves as the ligand binding site of the integrin head. In the integrins that lack the I-domain in their α subunit, the ligand binding site of the head is formed by the structural contribution of both α and β subunits (Xiong et al., 2001; Gahmberg et al., 2009). RGD-binding integrins, such as integrins that bind to fibronectin and laminin-binding integrins, are among non-I-domain integrins (Takada, 2007). There is a metal binding adhesion site (MIDAS) in the head part of β subunit of non-I-domain integrins which binds to divalent metal cations such as magnesium (Gahmberg et al., 2009). The involvement of this cation binding has been shown to be important for integrin binding to RGD ligands (Zhu et al., 2008). Not much is known about the transmembrane part of the integrins (Gahmberg et al., 2009). It has been shown that the transmembrane domain contains a conserved motif which is important in α and β heterodimerization (Kim et al., 2009). The cytoplasmic tails of integrins are generally short (except for integrin β4 that has a long tail) with no enzymatic activity (Gahmberg et al., 2009). They, however, recruit several signaling molecules and adaptor proteins through which integrins become connected to different signaling pathways and the cytoskeleton (Gahmberg et al., 2009).

1.3.4. Integrin activation and signaling

The affinity of integrins for their extracellular ligands changes through activation and inactivation of integrins (Harburger and Calderwood, 2009). A well-studied model of integrin activation is αvβ3 integrin in which a bent position of extracellular head of integrin towards the plasma membrane makes the ligand binding site inaccessible for the ligand. The bent position, therefore, keeps the integrin in an inactive state. Integrin activation, however, is associated with straightening of the extracellular head and separation of integrin legs, transmembrane domain, and cytoplasmic tails (Humphries et al., 2003). It should be pointed out that it is controversial whether integrins generally need to straighten out to be active, although the necessity for conformational changes leading to opening up of the ligand binding site in the extracellular head of integrins is certain (Gahmberg et al., 2009). The conformational changes that lead to integrin activation may be initiated by outside-in signaling events from conformational changes in the

head to the separation of other parts of integrins, or by inside-out signaling events from the separation of cytoplasmic tails to opening up of the ligand binding sites in the head (Gahmberg et al., 2009). It has been established that talin – a cytoplasmic protein – has a key role in integrin activation (Harburger and Calderwood, 2009). Talin interacts with both the cytoplasmic tail of β subunit and the cytoskeleton, and initiates inside-out signaling which results in the propagation of conformational changes across the plasma membrane to the extracellular domains of integrin and thereby increasing their affinity for the ligands (Harburger and Calderwood, 2009). Other factors, such as the proteins of kindlin family, may also cooperate with talin in integrin activation (Larjava et al., 2008; Harburger and Calderwood, 2009). Moreover, the phosphorylation of cytoplasmic domain of the β subunit by different components of signaling pathways (e.g., signals from G-protein coupled receptors) could lead to conformational changes leading to integrin activation and increasing the affinity of integrins for their ligands (Takada, 2007; Harburger and Calderwood, 2009). Integrin interaction with extracellular ligands can lead to clustering of integrins and formation of small focal contacts. These small focal contacts can turn into larger focal adhesions. Integrin engagement in the focal contacts can initiate the outside-in signaling and interaction of cytoplasmic tail of integrin with the intracellular signaling molecules. These signaling molecules include enzymes, such as focal adhesion kinase (FAK) (Mitra et al., 2005), src family kinases (Ginsberg et al., 2005; Giannone and Sheetz, 2006), integrin-linked kinase (ILK) (Legate et al., 2006), and the small GTPases Ras and Rho (Takada, 2007), and adaptor proteins, such as paxillin and vinculin (Deakin and Turner, 2008; Harburger and Calderwood, 2009). The extent of outside-in signaling at focal adhesions is determined by the affinity of an integrin for its ligands and its valency which is the ability to undergo clustering and, therefore, increasing the number of individual integrin interactions with extracellular ligands (Gahmberg, 2009). Inside-out signaling begins from non-integrin cell surface receptors (such as growth factor receptors) or cytoplasmic molecules activating signaling pathways which would lead to the activation or deactivation of integrins (Gahmberg, 2009). Inside-out signaling may regulate integrin affinity to extracellular ligands through both conformational changes in the integrins and valency changes (integrin clustering). Both signaling events (outside-in and inside-out signaling) may occur at the same time or at different occasions during wound healing and other physiological and pathological conditions (Gahmberg, 2009). The disassembly of integrins from their ligands – called integrin disengagement – is a necessary step for the termination of

signaling events or facilitating cell spreading and migration (Harburger and Calderwood, 2009). Physical forces as well as the dissociation of the proteins from the adhesion complex through competition, phosphorylation or proteolysis could all participate in integrin disengagement (Harburger and Calderwood, 2009). It is noteworthy that integrin trafficking through clathrin-dependent and –independent internalization pathways has been linked to the regulation of intracellular signaling pathways (*e.g.*, Rho GTPase signaling) during processes such as cytokinesis and cell migration (Caswell et al., 2009).

1.3.5. Keratinocytes integrins in resting adult epithelium

In resting epithelium, integrins are responsible for maintaining the epithelial cell homeostasis (*i.e.*, proliferation and differentiation), and for the attachment of basal keratinocytes to the basement membrane and the underlying connective tissue through hemidesmosomes (Watt and Jones, 1993; Watt, 2002; Santoro and Gaudino, 2005). The expression of the integrins in the resting epithelium is usually confined to the basal cells (Watt, 2002).

Keratinocytes in healthy basal epithelia express α6β4, α3β1, α2β1, and α9β1 integrins (Larjava et al., 1996; Häkkinen et al., 2000b). The α6β4 integrin is an essential part of hemidesmosomes (Geuijen and Sonnenberg, 2002). Both α6β4 and α3β1 integrins bind to laminin-332 in the basement membrane and support the integrity of the epithelium (Parks, 2007). The suppression of α6, β4, α3, and β1 integrin subunits, or LM-5 in gene-targeted mice has been associated with keratinocyte detachment from the basement membrane and blistering of skin (DiPersio et al., 1997; Ryan et al., 1999; Raghavan et al., 2000; Raymond et al., 2005; Häkkinen et al., 2000a). These data indicate the important role of α6β4 and α3β1 integrins in supporting of basal cell interactions with the basement membrane and its underlying matrix. The involvement of α3β1 integrin in cell differentiation has also been shown (Symington and Carter, 1995). It has been demonstrated that α2β1 together with α3β1 integrins mostly localize in basolateral and apical aspects of the keratinocytes suggesting a role for these integrins in cell-cell adhesion (Symington et al., 1993). The α2β1 integrin is the main collagen receptor in keratinocytes with high affinity for type I and III fibrillar collagens –the molecules that are not in contact to keratinocytes in intact skin or mucosa (Parks, 2007). The α2β1 integrin is more abundantly expressed in keratinocytes than α3β1 integrin, although a clear function has not been described

for this integrin in the intact epidermis (Parks, 2007). The tenascin-C-binding α9β1 integrin is also expressed against the basement membrane in the resting basal epithelium (Larjava et al., 1996; Häkkinen et al., 2000b). However, the function of this integrin in the intact epithelia of skin and mucosa is not clear as its main binding partner, tenascin-C, is not a part of the basement membrane (Larjava et al., 1996).

1.3.6. Changes in keratinocytes integrin expression during wound healing

Wound provisional matrix contains molecules that are not normally in contact with resting keratinocytes. These molecules include fibronectin, vitronectin, tenascin-C, and several types of collagen (Gailit and Clark, 1994; Larjava et al., 1996). Moreover, keratinocytes deposit LM-332, but not other components of the basement membrane, in the wound bed as they migrate (Larjava et al., 1996; Nguyen et al., 2000; Santoro and Gaudino, 2005). Wound keratinocytes change their integrin profile through upregulation, re-distribution, and neo-expression of integrins that are necessary for migration and re-epithelialization (Clark 2001; Larjava et al., 2002; Martin and Parkhurst, 2004; Hantash et al., 2008). Migratory keratinocytes express several integrins during wound healing. These integrins include α5β1, α3β1, α6β4, α2β1, α9β1, αvβ1, αvβ5, and αvβ6 integrins (Larjava et al., 1996; Häkkinen et al., 2000b; Santoro and Gaudino, 2005).

During wound healing, α6β4 integrin is redistributed from basal locations (*i.e.*, hemidesmosomes) to a more diffuse localization which promotes detachment of keratinocytes from the basement membrane and a migratory phenotype (Kurpakus et al., 1991). Considering the presence of LM-332 in the wound provisional matrix (Larjava et al., 1993), the presence of α6β4 integrin may be necessary for later formation of new hemidesmosomes as new basement membrane is formed. The diffuse expression of α6β4 integrin is associated with the upregulation and localization of α3β1 integrin in the focal contacts at the basal surface promoting spreading and migration of keratinocytes on LM-332 (Larjava et al., 1996; Kreidberg, 2000; Parks, 2007). Similarly α2β1 integrin is both stimulated and re-located from cell-cell contacts to the basal surface of the migrating keratinocytes (Häkkinen et al., 2000a). The expression of α2β1 integrin enables the migrating keratinocytes to move over different types of collagen which are exposed due to the wound (Pilcher et al., 1997; Zhang et al., 2006). The α2β1 integrin is also involved in

the degradation of collagens through the induction of matrix metalloproteinase-1 (MMP-1) (Larjava et al., 1996; Pilcher et al., 1997; Dumin et al., 2001). The expression of α9β1 integrin is also upregulated during wound healing. This upregulation has been shown to coincide with the deposition of tenascin-C (the main ligand for α9β1 integrin) underneath the wound keratinocytes and to be crucial for the keratinocyte proliferation and proper re-epithelialization (Häkkinen et al., 2000a; Singh et al., 2009).

Vitronectin-binding αvβ5, along with fibronectin-binding integrins αvβ1, αvβ6, and α5β1 are not normally expressed in resting epithelium of skin and mucosa (except for the constitutive expression of the αvβ6 integrin in the skin hair follicles and the junctional epithelium of gingiva as shown by Xie et al., 2009 and Ghannad et al., 2008, respectively). The expression of these integrins is, however, induced when keratinocytes are activated (e.g., in cell culture or during wound healing) (Larjava et al., 1993; Haapasalmi et al., 1996; Häkkinen et al., 2000a). The αvβ5 integrin is a main receptor for vitronectin not normally expressed in resting epithelium. Its expression, however, is induced in cultured keratinocytes and in migrating wound keratinocytes in pig (Gailit et al., 1994; Clark et al., 1996), but has not been detected in human oral mucosal wounds (Larjava et al., 1996). The αvβ1 integrin is a weak fibronectin receptor which mediates cell adhesion, but not cell migration, on fibronectin (Zhang et al., 1993). The exact role of αvβ1 is not known. The αvβ6 and α5β1 integrins are the main fibronectin receptors during wound healing (Larjava et al., 1993; Haapasalmi et al, 1996; Häkkinen et al., 2000a). The α5β1 integrin appears in early stages of wound healing suggesting its role in keratinocyte migration, while the maximal expression of αvβ6 integrin coincides with the basement membrane organization and wound closure (Haapasalmi et al, 1996; Larjava et al., 1993; Larjava et al., 1996). The exact role of αvβ6 integrin during wound healing is yet to be clarified; however, our present knowledge of αvβ6 integrin and its implications in wound healing are discussed in the following sections.

1.4. Integrin αvβ6

1.4.1. Biology of integrin αvβ6

The αvβ6 integrin is an epithelial cell surface receptor which is primarily expressed during development (Breuss et al., 1995). In adulthood, αvβ6 integrin expression is none or

minimal in the epithelia with the exception of hair follicles and the junctional epithelium of gingiva that constitutively express this integrin (Breuss et al., 1995; Ghannad et al., 2008; Xie et al., 2009). The expression of αvβ6 integrin is highly induced during tissue remodeling associated with endometrial cycle, wound healing, carcinomas, and in certain inflammatory conditions (Breuss et al., 1995; Huang et al., 1996; Hahm et al., 2007; Vigneault et al., 2007). The ITGB6 gene is located on chromosome 2 in humans (Krissansen et al., 1992). Comparing sequence identity of ITGB6 gene in human and guinea pig showed 94% cross-species conservation. The cytoplasmic domain of β6 integrin subunit includes an 11-amino acid C-terminal part which is not shared by other β integrin subunits; a characteristic that is clearly distinguished from other β subunits (Sheppard et al, 1990). The β6 integrin subunit only pairs with the αv subunit forming the complex of αvβ6 integrin (Hynes, 2002). Like all other αv integrins, αvβ6 integrin binds to its ligands through the RGD motif which is a tripeptide recognition sequence composing of arginine, glycine, and aspartic acid (Thomas et al., 2006). Fibronectin, tenascin-C and vitronectin are all binding partners of the αvβ6 integrin in the ECM (Busk et al., 1992; Prieto et al., 1993; Huang et al., 1998). The αvβ6 integrin also binds to the RGD sequence of latency-associated peptides 1 and 3 (LAP-1 and LAP-3), which are the N-terminal parts of the latent molecules of TGF-β1 and TGF-β3 (Munger et al., 1999; Annes et al., 2002). Moreover, the αvβ6 integrin is a binding partner for the viral capsids of the foot and mouth disease virus (FMDV) and Coxsackie virus 9 (CAV-9) (Miller et al., 2001; Williams et al., 2004). The binding partners of the αvβ6 integrin are summarized in Table 1.1.

Table 1.1. Ligands of αvβ6 integrin

Ligand	Reference
Fibronectin	Busk et al., 1992
Tenascin-C	Prieto et al., 1993
Vitronectin	Huang et al., 1998
LAP of TGF-β1	Munger et al., 1999
LAP of TGF-β3	Annes et al., 2002
Foot and mouth disease virus (FMDV)	Miller et al., 2001
Coxsackie virus (CAV-9)	Williams et al., 2004

Mice which lack the β6 integrin gene have no abnormality in terms of development or reproduction, although they produce significant infiltration of inflammatory cells in their skin and lungs, in addition to chronic inflammatory periodontal disease (Huang et al., 1996; Ghannad et al., 2008). An increased expression of the αvβ6 integrin has been associated with several cellular functions including cell migration, cell proliferation, cell survival, activation of TGF-βs, viral entry and infectivity, modulation of protease activity, and invasiveness of carcinoma cells (Miller et al., 2001; Williams et al., 2004; Thomas et al., 2006). In the following sections, those cellular functions of the αvβ6 integrin that have been discovered so far will be discussed. Moreover, the up-to-date findings regarding the possible roles of the αvβ6 integrin during wound healing and carcinogenesis will be reviewed.

1.4.2. Cellular functions of αvβ6 integrin: *in vitro* and *in vivo* implications

1.4.2.1. The αvβ6 integrin-induced promotion of cell spreading and migration, *in vitro*

Among other fibronectin receptors, the αvβ6 integrin has been shown to be critical in spreading and haptotactic motility of HaCaT keratinocytes on fibronectin- an important component of the wound provisional matrix (Koivisto et al., 1999). Nevertheless, in HaCaT keratinocytes, αvβ6 integrin showed only a minor role in the lateral migration of the cells on

fibronectin (Koivisto et al., 1999). A comparison between the β6 integrin deficient (β6-/-) versus wild-type mouse keratinocytes, however, suggested a more significant role for the αvβ6 integrin in the keratinocyte migration on two ligands of this integrin, fibronectin and vitronectin (Huang et al., 1998). The αvβ6 integrin-mediated migration of mouse keratinocytes on fibronectin was considerably augmented by the hepatocyte growth factor through a mechanism that involved protein kinase C (PKC) (Huang et al., 1998). *In vitro* studies have shown that β6 subunit potently accumulated at the leading edge of the migrating cells on fibronectin, suggesting a role for αvβ6 integrin in cell migration (Huang et al., 1998). It has been found that in human primary oral keratinocytes αvβ6 integrin promotes cell migration on fibronectin, resulting in the upregulation of the pro-enzyme form of MMP-9. The activation of MMP-9 pro-enzyme will further increase keratinocytes migration (Thomas et al., 2001a). The localization of MMP-9 to the αv subunit in the foot-like cell membrane protrusions (filopodia) suggests a facilitating role for this protease in cell motility (Thomas et al., 2006). Furthermore, TNF-α has been shown to induce the αvβ6 integrin expression, which, in turn, resulted in an αvβ6 integrin–dependent increase in keratinocyte migration and MMP-9 secretion (Scott et al., 2004).

Taken together, most *in vitro* studies suggest a role for the αvβ6 integrin in promoting cell spreading and migration, although some studies have failed to find such role. It is important to note that the *in vitro* data should be further evaluated and interpreted in the context of different *in vivo* conditions, such as wound healing and carcinogenesis. This will be discussed in sections 1.4.3 and 1.4.4.

1.4.2.2. The αvβ6 integrin-induced suppression of apoptosis, *in vitro*

It has been shown that the *de novo* expression of the αvβ6 integrin in oral squamous cell carcinoma protects the cells from anoikis, which is defined as programmed cell death in the conditions that the cells are not appropriately anchored to their surrounding ECM (Janes and Watt, 2004). This anti-apoptotic effect of αvβ6 integrin in oral squamous cell carcinoma cells is mediated through protein kinase-B (PKB)/Akt activation (Janes and Watt, 2004). This effect could potentially be important in tumor progression where tumor cells should grow and survive in the absence of a basement membrane. It is not clear, however, whether non-transformed

keratinocytes show the same effect in other physiological conditions, such as wound healing, where αvβ6 integrin expression is induced.

1.4.2.3. The αvβ6 integrin and activation of TGF-βs

Based on *in vitro* studies, the binding of the αvβ6 integrin to the RGD motif of LAP-1 and LAP-3 can result in conformational changes and the activation of the latent molecules of TGF-β1 and TGF-β3 (Annes et al., 2003, 2004). TGF-β2 does not contain an RGD sequence and therefore is not activated by the αvβ6 integrin (Annes et al., 2002). Both the extracellular domains and the cytoplasmic tail of the αvβ6 integrin are important in the activation of TGF-βs, since counter-traction forces from both cytoskeleton and ECM result in the conformational changes in the latent molecule of TGF-βs and their activation (Keski-Oja, 2004). Mechanisms of TGF-β activation including the integrin-mediated activation of TGF-β are further discussed later in this chapter. *In vitro* studies showing the αvβ6 integrin-mediated activation of TGF-βs have been supported by several *in vivo* findings that are derived from experiments performed in mice. It has been shown that mice deficient in the β6 integrin subunit (β6-/- mice) develop skin and lung inflammation similar to that of TGF-β1-null mice. They also develop late-onset emphysema due to the lack of TGF-β inhibitory effect on MMP-12 expression by alveolar macrophages (Huang et al., 1996; Morris et al., 2003). Double-knockout mice for both β6 integrin and thrombospondin-1 (TSP-1, another activator of TGF-β) resembled more characteristics of TGF-β1-null mice inflammatory phenotype, including multi-organ inflammation (Ludlow et al., 2005). Furthermore, β6-/- mice developed classic signs of chronic inflammatory periodontal disease possibly due to lack of the αvβ6 integrin-mediated activation of TGF-β1 (Ghannad et al., 2008). β6-/- mice are also protected from TGF-β1-mediated lung fibrosis induced by bleomycin treatment or radiation (Horan et al., 2008; Puthawala et al., 2008). Moreover, genetic ablation of the β6 subunit as well as β6 blockade using β6 monoclonal antibodies had protective effects against TGF-β1-mediated renal and hepatic fibrosis (Hahm et al., 2007; Wang et al., 2007).

In summary, *in vivo* studies suggest a role for the αvβ6 integrin-mediated activation of TGF-β1 in suppressing certain inflammatory conditions and in protecting different organs from

fibrosis (Huang et al., 1996; Morris et al., 2003; Hahm et al., 2007; Wang et al., 2007; Horan et al., 2008; Puthawala et al., 2008).

1.4.2.4. The αvβ6 integrin endocytosis upon ligand binding

It has been shown that the binding of the αvβ6 integrin to some of its ligands, including the viral capsid of FMDV, induces endocytosis of the integrin-ligand complexes (Weinreb et al., 2004; Berryman et al., 2005). FMDV is the etiological agent of a severe vesicular disease of cloven-hoofed animals, called foot-and-mouth disease. It has been shown that FMDV applies αvβ6 integrin as its main cell entry receptor in epithelial cells. FMDV gets internalized together with αvβ6 integrin through the clathrin-dependent internalization pathway (Berryman et al., 2005). This way the virus is delivered to the sites where it can produce infectivity. CAV-9 is a significant human pathogen which also uses αvβ6 integrin as its primary receptor. Internalization of CAV-9 has been shown to follow an endocytotic pathway that is dependent on the αvβ6 integrin and some other endocytotic molecules, but not on clathrin or caveolin-1 (Heikkilä et al., 2010). Ligand-mimetic β6 integrin function-blocking antibodies, which contain an RGD motif recognized by the αvβ6 integrin, as well as LAP are other ligands that bind to and get internalized together with αvβ6 integrin (Weinreb et al., 2004).

1.4.3. The αvβ6 integrin and wound healing

The neo-expression of the αvβ6 integrin in wound keratinocytes is confined to the basal cell layer in the skin. It is, however, detected in basal and suprabasal keratinocytes in oral mucosal wounds (Jones et al., 1993, 1997; Häkkinen et al., 2000b). The exact regulatory factors that result in the temporary high expression of αvβ6 integrin during later stages of wound healing (when epithelium completely covers the wound bed) are not well understood. When colon carcinoma cells are cultured in high density allowing the cells to establish abundant cell-cell contacts with each other, αvβ6 integrin expression is also increased (Niu et al., 2001). Therefore, the peak expression of αvβ6 integrin after the restoration of epithelial integrity during wound healing may also depend on reestablishment of the cell-cell contacts in the wound epithelium (Haapasalmi et al., 1996; Häkkinen et al., 2000b). It has been shown that TGF-β1 upregulates the neoexpression of the αvβ6 integrin in normal keratinocytes and in non-transformed HaCaT

keratinocytes (Zambruno et al., 1995; Koivisto et al., 1999). TNF-α has also been recognized as an inducer of αvβ6 integrin expression in keratinocytes (Scott et al., 2004). It has been found that TGF-β1 and TNF-α have a synergistic effect on the upregulation of the αvβ6 integrin in colon carcinoma cells (Bates et al., 2005). Since both of these cytokines are highly detected during wound healing, their synergistic effect may also play a role in the induction of αvβ6 integrin (Barrientos et al., 2008). More investigation is required to unveil the regulatory mechanisms underlying the induction and downregulation of the αvβ6 integrin during wound healing. The role of the αvβ6 integrin during wound healing is also largely unknown.

It has been shown that αvβ6 integrin facilitates cell migration and adhesion on fibronectin, TN-C, and vitronectin, which are all components of the early wound provisional matrix. Moreover, αvβ6-dependent upregulation of MMP-9 has been shown to facilitate cell detachment from the basement membrane and therefore promote cell motility (Thomas et al., 2001a). These *in vitro* findings suggest a potential facilitating role for the αvβ6 integrin in keratinocyte migration in early wound healing. However, although the β6 mRNA is detectable in keratinocytes at the migrating wound edge, the uniformly more strong expression of the αvβ6 integrin occurs at day -3 post-wounding and the maximal expression is relatively late when the epithelial sheets have joined (Haapasalmi et al., 1996; Häkkinen et al., 2004). This suggests a more important role for the αvβ6 integrin in the later stages of wound healing. In β6 integrin over-expressing transgenic mice in which β6 integrin is highly expressed both in intact skin and from the very first stages of wound healing, both skin architecture and wound repair remained normal (Häkkinen et al., 2004). In a stressed condition, however, mice over-expressing β6 integrin produced chronic fibrotic wounds with a high content of TGF-β1 (Häkkinen et al., 2004). Our laboratory has also previously found that human chronic wounds show a prolonged expression of the β6 integrin (Häkkinen et al., 2004). It has been shown that immunosuppression associated with the old age and glucocorticoid treatment led to an imbalance of the TGF-β1 activity and impaired wound healing (Wu et al., 1999; Beer et al., 2000; Gosain and Dipietro, 2004). Interestingly, the β6 integrin over-expressing mice that were both old and under hydrocortisone treatment showed improved healing response, the effect that could be due to a compensatory β6 integrin-mediated activation of TGF-β1 (Aldahlawi et al., 2006). On the other

hand, Xie et al. (2009) have found that β6-/- mice challenged with Dexamethazone showed accelerated wound healing that was characterized with an enhanced keratinocyte proliferation observed in the wound samples possibly as a result of reduced TGF-β1 activation and its inhibitory effect on the cell growth. Although these data do not propose a role for β6 integrin in normal wound healing, they are suggestive of a role for the β6 integrin in the pathophysiology of impaired wound repair via the αvβ6 integrin-mediated modulation of TGF-β1 activity. The exact role of the αvβ6 integrin during normal and abnormal wound healing especially in regards to the αvβ6 integrin-mediated regulation of TGF-β1 and TGF-β3 has yet to be further investigated.

1.4.4. The αvβ6 integrin and carcinogenesis

There are many similarities between the biological processes of wound healing and carcinogenesis to the extent that carcinogenesis could be described as a misregulated wound healing process (Dvorak, 1986; Riss et al., 2006). In both processes migrating epithelial cells at the front edge should go through well-controlled changes that enable them to detach from their basement membrane or surrounding ECM, be motile, and reside in the new location to promote healing or tissue invasion (Thomas et al., 2006). Many of the αvβ6 integrin ligands from the ECM are found and upregulated in both wound healing and carcinogenesis processes (Chiquet-Ehrismann and Chiquet, 2003). The *de novo* expression of the αvβ6 integrin has been reported in many types of carcinomas including oral and skin squamous cell carcinoma (Hamidi et al., 2000; Impola et al., 2004; Thomas et al., 2006). Unlike the temporary expression of the αvβ6 integrin in wound healing, the expression of this integrin in carcinomas seems to be permanently turned on, suggesting a role for the αvβ6 integrin in tumor progression and invasiveness (Thomas et al., 2006). As discussed earlier, most of the *in vitro* studies have suggested that αvβ6 integrin promotes epithelial cell motility and migration on its ligands. Accordingly, the expression of the αvβ6 integrin has been associated with epithelial-to-mesenchymal transition (EMT), a process that results in more motile and aggressive phenotype in carcinoma cells (Bates et al., 2005; Ramos et al., 2009). It has also been found that the αvβ6 integrin sustains tumor cell proliferation in colon carcinoma, both *in vitro* and *in vivo*, through the upregulation of its own expression via a protein kinase C (PKC)-mediated signaling pathway (Niu et al., 2001; Agrez et al., 1994). Moreover, the αvβ6 integrin-mediated upregulation of proteases may facilitate tumor cell

motility, migration, and invasiveness. It has been shown that the expression of αvβ6 integrin in oral squamous cell carcinoma (OSCC) cells resulted in the upregulation of MMP-3 and MMP-9 and promoted cell invasiveness both *in vitro* and *in vivo* (Thomas et al., 2001a, b; Ramos et al., 2002). However, the αvβ6 integrin-mediated inhibitory effect on the expression of MMP-13 did not change the OSCC invasiveness (Ylipalosaari et al., 2005). The upregulation of uPA, MMP-3, and MMP-9 in OSCC cells transfected by β6 construct, as shown by Dang et al., 2004 suggested the effectiveness of this protease cascade in promoting invasive behavior of the OSCC cells. Interestingly, it has been shown that the clathrin-dependent internalization of the αvβ6 integrin bound to an intracellular adaptor protein -called HCLS1-associated protein X1 (HAX1)- was a requirement for tumor cell invasion in the OSCC cells (Ramsay et al., 2007).

Taken together, the neo-expression of the αvβ6 integrin in many carcinoma cells, especially the carcinomas with more invasive phenotype or in metastatic stage, the role of this integrin in promoting cell migration, motility, and proliferation, as well as the αvβ6 integrin-mediated upregulation of different proteases, all suggest an important role for the αvβ6 integrin in tumor progression and invasiveness.

1.4.5. The αvβ6 integrin and clinical implications

The αvβ6 integrin has all the characteristics of being a good target for therapeutic purposes. It is expressed on the cell surface with minimal or no expression in normal adult tissue (except for the hair follicles and the junctional epithelium of gingiva in which αvβ6 integrin is constitutively expressed, Ghannad et al., 2008; Xie et al., 2009). Moreover, its expression is induced at a level significantly higher than the surrounding normal tissue during conditions such as wound healing, many fibrotic conditions, and carcinogenesis (Thomas et al., 2006). The β6 integrin-mediated activation of TGF-β1 has been shown to play a key role in the pathogenesis of several TGF-β1-induced fibrotic conditions as shown in β6 integrin knockout mice (Horan et al., 2008; Puthawala et al., 2008). The therapeutic targeting of a locally expressed molecule such as the αvβ6 integrin instead of a multi-functional growth factor such as TGF-β1 is much preferred in managing the TGF-β1-mediated pathological conditions. Interestingly, the *in vivo* application of specific monoclonal antibodies to the αvβ6 integrin and small molecule αvβ6 inhibitors in rodents have been promising in the therapeutic management of acute biliary and portal liver

fibrosis (Wang et al., 2007; Popov et al., 2008). Monoclonal antibodies against αvβ6 integrin have been also used to prevent TGF-β1 mediated-pulmonary and -renal fibrosis (Hahm et al., 2007; Horan et al., 2008; Puthawala et al., 2008). It is conceivable, therefore, to expect promising results in the prevention or management of other TGF-β1-mediated fibrotic conditions, such as fibroproliferative scars, by blocking the β6 integrin. The β6 integrin function-blocking antibodies have been safely used in animal models. However, blocking the αvβ6 integrin in the junctional epithelium of gingiva in rat has been associated with the initiation of inflammatory periodontal disease due to suppression of αvβ6 integrin-mediated activation of anti-inflammatory TGF-β1 (Ghannad et al., 2008). Therapeutic targeting of αvβ6 integrin has also some experimental support in the management of carcinomas. It has been shown that the combined injection of the OSCC cells and anti-αvβ6 integrin antibodies into the mouth floor of nude mice resulted in a reduced incidence of tumor formation by 60% (Xue et al., 2001). Delivering therapeutic genes to carcinoma cells, including OSCC, by viral vectors is another potential approach for cancer therapy (Liu et al., 2004). The idea is that using molecular modifications viral vectors recognize and become internalized through specific integrins, in this case αvβ6 integrin, and deliver therapeutic genes to the carcinoma cells (Thomas et al., 2006). More investigation is required to translate the aforementioned experiments into the clinical trials.

1.5. Transforming growth factor β

Transforming growth factor-βs (TGF-βs) are multi-functional cytokines that are involved in many physiological and pathological conditions (Massagué J, 1990; Hyytiäinen et al., 2004). They play several key roles in maintaining the homeostasis and regulating the function of epithelial, endothelial, immune, and mesenchymal cells both during development and in adulthood (Nishimura, 2009). There are three high homologic mammalian isoforms of TGF-β: TGF-β1, TGF-β2, and TGF-β3, each of which is the product of a distinct gene (Sheppard, 2006). These three widely expressed mammalian isoforms of TGF-β, when examined *in vitro*, share similar receptor complexes, activate similar signaling pathways, and result in very similar effects on cell behavior (Derynck and Zhang, 2003; Nishimura, 2009). *In vivo*, however, they produce very divergent functions as well as substantially different phenotypes as shown by isoform-specific knockout animal models (Hyytiäinen et al., 2004; Nishimura, 2009). It has been shown

that TGF-β1 knockout mice die shortly after birth due to widespread tissue inflammation and autoimmunity (Shull et al., 1992; Kulkarni et al., 1993). TGF-β2 knockout mice die around the time of birth, too, although mostly due to severe multi-organ developmental defects (Sanford et al., 1997). TGF-β3 knockout mice, on the other hand, survive but produce cleft palate (Kaartinen et al., 1995). TGF-β1, the most frequently expressed isoform of TGF-β, has been shown to be responsible for many of TGF-β-mediated physiological effects since its misregulation results in many pathological processes such as fibrotic conditions (O'Kane and Ferguson, 1997). For this reason, TGF-β1 has been the main subject of many studies for years, and most of the information presented here is representing this isoform of TGF-β.

1.5.1. Functions of TGF-β

In this section the main functions of TGF-β in cell proliferation, ECM composition, and immune system will be reviewed. These are the same functions that are also applicable during conditions such as wound healing.

One of the main functions of TGF-β is its inhibitory effect on epithelial cell growth. It has been shown that TGF-β regulates cell proliferation rate by decreasing the growth of epithelial cells while increasing the growth of mesenchymal cells (Tucker et al., 1984; Shipley et al., 1985; Robey et al., 1987; Sporn et al., 1987; Moses, 1992). Inhibition of epithelial cell proliferation by TGF-β1 involves downregulation of c-Myc, leading to the upregulation of cyclin-dependent kinase inhibitors p15 and p21, which inhibit the CDK4/6-cyclin D and CDK2-cyclin E-mediated phosphorylation of the retinoblastoma protein (Robson et al., 1999; ten Dijke et al., 2002; Arnold and Korc, 2005). As a matter of fact, this growth inhibitory effect of TGF-β1 is a protective mechanism against progression of carcinomas in their early stages, the effect that could be overcome by other mechanisms, including mutations in TGF-β1 signaling components, making TGF-β1 a tumor promoter in later stages of cancers (Derynck et al., 2001).

Another main function of TGF-β, especially TGF-β1 with its pro-fibrotic effects, is affecting the ECM synthesis. TGF-β1 induces the production and organization of ECM components such as collagen and fibronectin (Roberts et al., 1986; Keski-Oja et al., 1988). Moreover, TGF-β1 increases the expression of proteinase inhibitors (*e.g.*, tissue inhibitor of

metalloproteinases or TIMPs) and decreases the expression of matrix degrading proteinases such as MMPs (O'Kane and Ferguson, 1997). Through modulation of integrins, as the binding partners of ECM components on the cell surface, TGF-β can further regulate the ECM organization and – accordingly – the cellular behavior (Hyytiäinen et al., 2004).

TGF-β also plays different critical roles in the regulation of immune system. It is secreted by most of immune cells and regulates them in an autocrine or paracrine manner (Moustakas et al., 2002). TGF-β has inhibitory effects on the proliferation and differentiation of many of immune cells including B and T lymphocytes (Letterio and Roberts, 1998). It has been shown that TGF-β1 knockout mice die early due to widespread and massive infiltration of lymphocytes into their tissues and the over-production of autoantibodies (Shull et al., 1992; Kulkarni et al., 1993). This demonstrated the central role of TGF-β in inhibiting inflammation and autoimmune diseases. Suppression of TGF-β signaling specifically in T cells has been shown to be most important in the production of autoimmune diseases (Gorelik and Flavell, 2000). Other than its inhibitory effect on the proliferation of T cells, TGF-β specifically inhibits the differentiation of helper T cells (Th1 and Th2) and suppresses the activity of cytotoxic T cells (Li et al., 2006).

On the other hand, TGF-β has been known to be a potent chemo-attractant for some immune cells such as monocytes and macrophages (Bogdan and Nathan, 1993). TGF-β has been also suggested to promote monocytes to macrophage differentiation (Bombara and Ignotz, 1992).

Interestingly, it has been shown that TGF-β1 autoinduces its own expression and also stimulates the expression of other TGF-β isoforms, hence reinforcing their biological effects (O'Kane and Ferguson, 1997).

1.5.2. Structure of TGF-β

Mammalian isoforms of TGF-β are synthesized as homodimeric pro-TGF-β which is composed of a dimeric TGF-β peptide (called mature or active TGF-β). This mature TGF-β is covalently bound to a dimeric pro-peptide called latency associated peptide (LAP) which is enzymatically processed in the cells to produce small latent complex (SLC) of TGF-β consisting of the mature TGF-β that remains non-covalently associated to LAP (Hyytiäinen et al., 2004) (Figure 1.3). This association keeps the mature TGF-β inactive (Koli et al., 2001). Different mammalian isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) show sequence variations in

LAP portions; these variations are called LAP1, LAP2, and LAP3, respectively (Annes et al., 2003). Most commonly, SLC is covalently associated with members of larger proteins called latent TGF-β binding proteins (LTBPs) resulting in the formation of large latent complex (LLC) of TGF-β (Saharinen et al., 1999; Saharinen and Keski-Oja, 2000; Koli et al., 2001) (Figure 1.3). It has been shown that LLC is secreted from the cells more efficiently than SLC (Saharinen et al., 1999; Wipff and Hinz, 2008). There are four isoforms of LTBP, of which only three (LTBP-1, -3, and -4) could bind to the SLC of TGF-β isoforms, and from those three, only LTBP-1 is involved in the process of TGF-β activation (Ramirez and Pereira, 1999; Saharinen et al., 1999; Saharinen and Keski-Oja, 2000; Annes et al., 2004). This will be discussed in more details in the following sections.

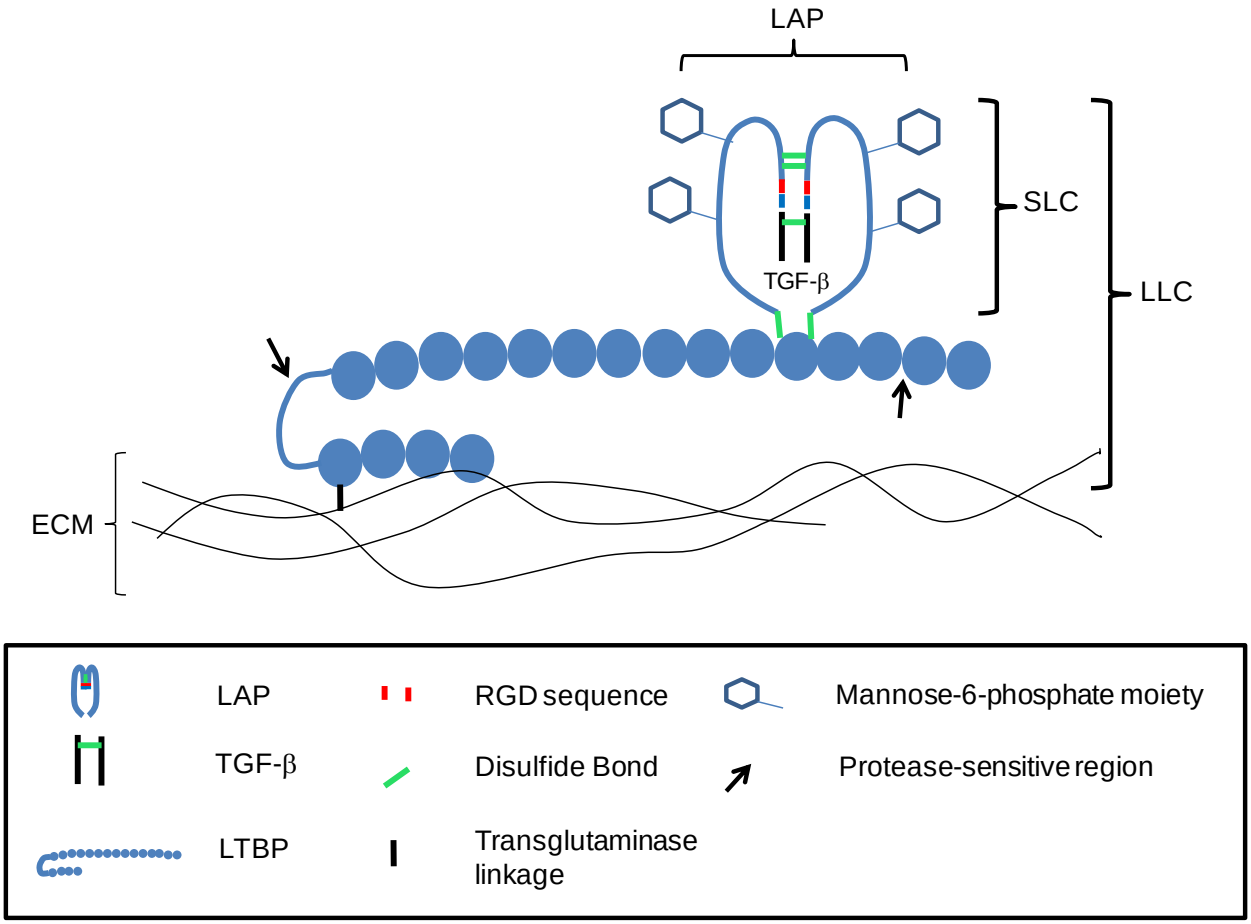

Figure 1.3. Schematic structure of small and large latent complexes of TGF-β (SLC & LLC); (modified from Wipff and Hinz, 2008)

Latent TGF-β is incorporated and stored in the ECM of most adult mammalian tissues through direct binding of LTBPs with several ECM proteins such as fibrillin-1, fibronectin, and vitronectin, the process that is mediated by the action of the extracellular enzyme tissue transglutaminase (ten Dijke and Arthur, 2007; Taipale et al., 1994; Schoppet et al., 2002; Nunes et al., 1997). The storage of the pre-formed latent TGF-β allows cellular rapid response to the presence of TGF-β activators and environmental changes (Wipff and Hinz, 2008).

1.5.3. Mechanisms of TGF-β activation

To be functional, the latent molecule of TGF-β should be activated and the mature molecule of TGF-β should be either proteolytically liberated from its pro-peptide or be exposed to its neighboring receptors by conformational changes in the latent molecule (Koli et al., 2001; Annes et al., 2003). TGF-β has been known to have several activators that dissociate it from its pro-peptide and convert it into the active form, as follows.

1.5.3.1. Integrin-independent mechanism of TGF-β activation

In vitro, latent TGF-β can be activated by different factors that cause protein denaturation and conformational changes such as extreme temperatures or pH, as well as oxidants and ionizing radiation (Brown et al., 1990; Barcellos-Hoff et al., 1994, 1996). *In vitro* application of glycosidases, which remove carbohydrates from the latent complex and compromise the stability of the SLC, has also been shown to cause TGF-β activation (Miyazono and Heldin, 1989; Schultz-Cherry and Hinshaw, 1996). The *in vivo* role of those activators, however, is not clear (Hyytiäinen et al., 2004). Different proteases, such as MMPs (MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13), plasmin, urokinase-type and tissue-type plasminogen activators, can also cleave LAP and release mature TGF-β (D'Angelo et al., 2001; Maeda et al., 2002; Yu and Stamenkovic, 2000; Mu et al., 2002; Lyons et al., 1990; Chu and Kawinski, 1998; Nunes et al., 1995). Mannose-6-phosphate receptor has also been shown to promote TGF-β activation through binding to and bringing together both latent TGF-β and its proteases activators (Godár et al., 1999; Yang et al., 2000). Moreover, the extracellular matrix molecule thrombospondin 1 (TSP-1) has been found to activate TGF-β both *in vitro* and *in vivo* through conformational changes (Shultz-Cherry and Murphy-Ullrich, 1993; Crawford et al., 1998). As a matter of fact, TSP-1

and the integrins αvβ6 and αvβ8 are the only factors whose involvement in TGF-β activation has been conclusively shown in animal models (Crawford et al., 1998; Munger et al., 1999; Travis et al., 2007).

1.5.3.2. Integrin-dependent mechanism of TGF-β activation

It has been shown that all integrins that contain αv subunit (αvβ1, αvβ3, αvβ5, αvβ6, and αvβ8) in addition to the integrins α5β1 and α8β1 and also the platelet integrin αIIbβ3 could bind to the RGD motif in LAP1 or LAP3 of latent TGF-β1 and TGF-β3, respectively (Munger et al., 1998; Lu et al., 2002; Ludbrook et al., 2003; Annes et al., 2004; Asano et al., 2005a,b; Wipff et al., 2007; Araya et al., 2006; Travis et al., 2007; Wipff and Hinz, 2008). Among those integrins, αvβ6 and αvβ8 integrins that bind to TGF-β with high affinity have been shown to efficiently activate TGF-β (Munger et al., 1999; Mu et al., 2002). Other integrins with low affinity interactions either are weak activators of TGF-β (*e.g.*, αvβ3 and αvβ5) or do not activate it at all (Nishimura, 2009). It seems that integrins are the main activators of TGF-β *in vivo* since mice with a mutation on RGD motif (RGD mutated to RGE) recapitulated the TGF-β1-null mice phenotype (Yang et al., 2007). It has been shown that mice lacking the functions of both integrins αvβ6 and αvβ8, two well-defined integrin activators of TGF-β, rendered very similar abnormalities as in TGF-β1- and TGF-β3- null mice. Those abnormalities include misregulation of immune system leading to severe autoimmunity and lack of langerhans cells seen in TGF-β1-null mice, and development of cleft palate that is a characteristic of TGF-β3-null mice (Aluwihare et al., 2009).

The αvβ6 integrin-mediated activation of TGF-β

In vitro and *in vivo* evidence for the activation of TGF-β1 and TGF-β3 by αvβ6 integrin has been reviewed earlier in this chapter. Here, the mechanistic aspects of such activation will be discussed. The αvβ6 integrin activates TGF-β by conformational changes, independently from any proteolytic activity (Munger et al., 1999; Annes et al., 2004). It seems that αvβ6-mediated activation of TGF-β is dependent on three important factors. Firstly, it has been shown that purified integrins alone are unable to activate latent TGF-β, and the association of αvβ6 integrin (and other integrins which are considered as TGF-β activators) to the plasma membrane and its

interaction with the actin cytoskeleton are necessary for TGF-β activation (Munger et al., 1999; Annes et al., 2004). Factors that increase actin stress fiber formation and cell contraction could, therefore, potentially facilitate the αvβ6 integrin-mediated activation of TGF-β (Wipff and Hinz, 2008). For example, it has been shown that thrombin through binding to protease-activated receptor 1 (PAR1) stimulates Rho A (a G-protein-coupled receptor with well-known roles in mediating cell contraction). PAR1 sends signals to αvβ6 integrin through Rho A to enhance αvβ6 integrin-mediated activation of TGF-β (Jenkins et al., 2006, 2008). Secondly, binding of SLC to LTBP-1 (but not to other LTBPs that lack the ECM binding domain present in LTBP-1) and its incorporation into the ECM are pivotal requirements for efficient αvβ6-mediated activation of TGF-β1 and TGF-β3 (Annes et al., 2003; Annes et al., 2004). And lastly, the presence of an ECM that resists the mechanical forces generated by the cytoskeleton and transmitted to the LLC could support and promote the αvβ6 integrin-mediated conformational changes of latent TGF-β and hence its activation (Wipff and Hinz, 2008). For example, the presence of fibronectin (FN) in the ECM has been shown to promote LTBP-1 incorporation into the ECM and the αvβ6 integrin-mediated activation of TGF-β (Fontana et al., 2005). Based on these pieces of evidence, it has been proposed that binding of αvβ6 integrin to LAP portion of LLC that is fixed into the ECM, results in the production of counter-traction forces from the cell cytoskeleton and the ECM leading to conformational changes in the latent molecule of TGF-β and its activation (Keski-Oja, 2004) (Figure 1.4).

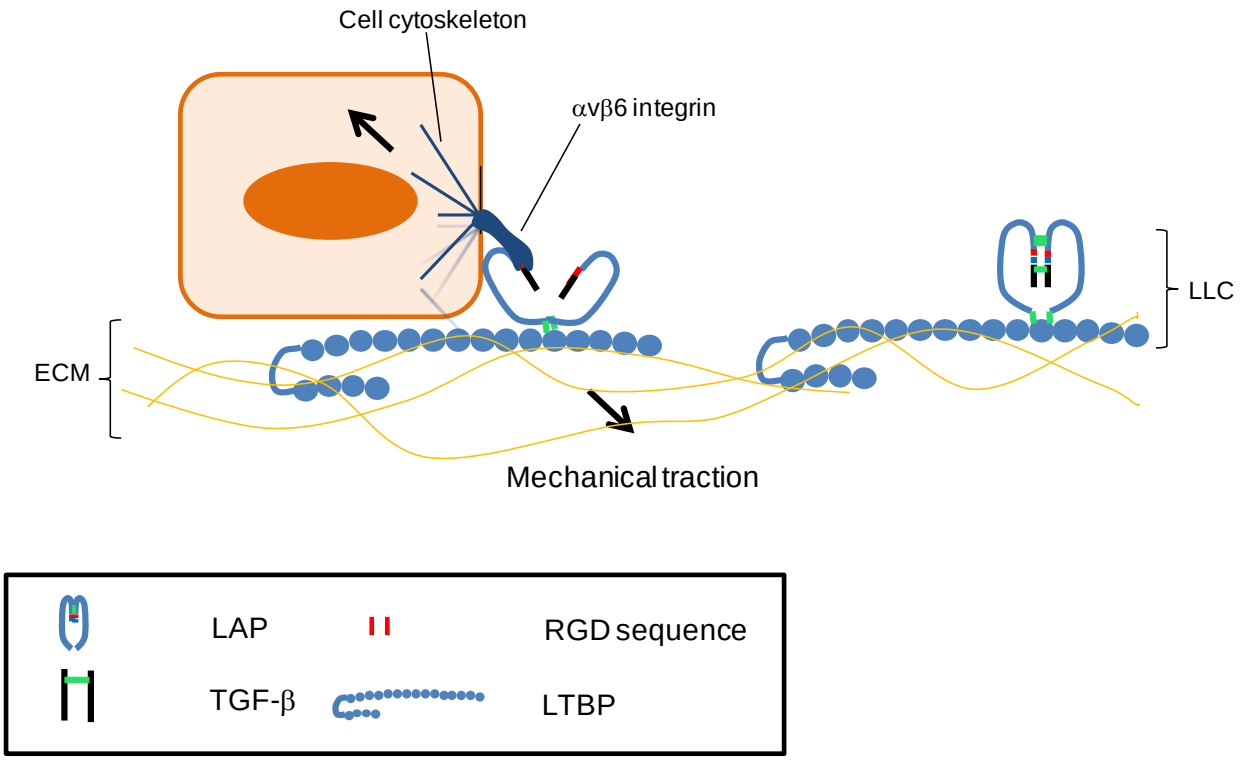

Figure 1.4. The αvβ6 integrin-mediated activation of TGF-β (modified from Nishimura, 2009)

It is most likely in this context that due to conformational changes, the mature TGF-β becomes exposed to its receptors on the same or neighboring cells while it remains attached to the LAP (Annes et al., 2002, 2004; Keski-Oja, 2004). This mechanism of activation allows for tight local regulation of TGF-β activity in the close vicinity of αvβ6 integrin-expressing cells.

Other integrin-mediated activation of TGF-β

Another potent activator of TGF-β, αvβ8 integrin, binds to LAP portion of TGF-β1 and TGF-β3 as does the αvβ6 integrin (Mu et al., 2002). However, αvβ8 integrin activates TGF-β through a totally different mechanism than αvβ6 integrin does. It has been shown that αvβ8 integrin binds to the latent TGF-β and presents it to the transmembrane protease MMP-14. Subsequently, MMP-14 cleaves the LAP and releases the mature TGF-β into the cell environment (Mu et al., 2002). This mechanism of activation does not require cell-to-cell contacts since the mature TGF-β is proteolytically liberated from the latent complex and could

target cells in both close and far distances (Sheppard, 2006). The αvβ8 integrin is expressed by a variety of cells including airway epithelial cells, fibroblasts, neuronal cells, and dendritic cells (Mu et al., 2002; Araya et al., 2006; Travis et al., 2007; Wipff and Hinz, 2008). The β8 null mice die early during embryonic life or soon after birth. These mice produce many fatal pathological abnormalities that are also observed in the TGF-β1 and/or TGF-β3 null mice. This shows the significance of the αvβ8 integrin role in the activation of TGF-β1 and TGF-β3, *in vivo* (Zhu et al., 2002).

A mechanism of activation close to αvβ8-mediated activation of TGF-β has been proposed for αvβ3 integrin that could serve as a docking site for latent TGF-β and MMP-9, or MMP-2 (Brooks et al., 1996; Rolli et al., 2003; Wipff et al, 2008). This close vicinity of protease activators of TGF-β and the latent molecule of TGF-β could potentially be important in the regulation of TGF-β activity. Moreover, αvβ3 integrin could cluster with TGF-βRII when stimulated by active TGF-β. This suggests that αvβ3 integrin increases the local availability of TGF-βRII to the active cytokine (Scaffidi et al., 2004; Galliher and Schiemann, 2006). It has been also shown that αvβ5 and possibly αvβ3 integrins could directly activate TGF-β in the absence of proteases in myofibroblasts (Wipff et al., 2007). Similar to the αvβ6 integrin-mediated activation of TGF-β, αvβ5 and αvβ3 integrins need contractile forces leading to conformational changes in the latent molecule of TGF-β and its direct activation (Wipff and Hinz, 2008). The *in vivo* significance of such mechanisms of TGF-β activation remains to be further investigated as the combined β3/β5 knockout mice have developed normally (Reynolds et al., 2002).

1.5.4. TGF-β signaling pathways

Upon activation, all three mammalian isoforms of TGF-β bind to TGF-β receptor type-II (TGF-βRII) which, in turn, joins to and activates TGF-β receptor type-I (TGF-βRI) at the cell surface (Massagué et al., 2000). They, then, signal through either smad-dependent or smad-independent pathways. Signaling through smad mediators is the best-recognized signaling pathway that is commonly used by TGF-βs (Derynck and Zhang, 2003; Feng and Derynck, 2005). The *in vivo* significance of smad-dependent pathway has been shown by severe and/or

lethal developmental defects caused by genetic deletion of TGF-β signaling mediators, smad2 and smad3, in mice (Nomura and Li, 1998; Waldrip et al., 1998; Zhu et al., 1998; Datto et al., 1999; Yang et al., 1999; Bonniaud et al., 2004). Upon the binding of TGF-β to its receptor complex, activated TGF-βRI phosphorylates receptor-regulated smads (R-smads: smad2 and/or smad3), which are important mediators of TGF-β signaling pathway (Massagué et al., 2000). R-smads are retained in the cytoplasm through binding to SARA (smad anchor for receptor activation) that occludes a nuclear import sequence on the smads. Moreover, SARA facilitates presenting R-smads to the activated receptors (Tsukazaki et al., 1998). Binding to the activated receptors decreases the affinity of R-smads for SARA, while increasing their affinity for their co-smad called smad4 (Xu et al., 2000). The whole smad complex, then, is translocated into the nucleus and binds to the smad response elements in the target genes promoter regions resulting in the modulation of potentially hundreds of TGF-β-induced genes (Massagué and Gomis, 2006). Alternatively, competitive interaction of inhibitory smads (I-smads), namely smad6 and smad7, could inhibit R-smad phosphorylation and recruit the components involved in downregulation of TGF-β receptor levels and function (Itoh and ten Dijke, 2007). The cellular context is an important factor in determining what smads (R-smads versus I-smads) would interact to TGF-β receptors and also what specific genes are induced by TGF-β signal transduction during different biological processes (Massagué and Gomis, 2006). Studies on TGF-β receptors have pointed out the importance of internalization pathways in TGF-β signaling. Interestingly, the internalization of activated TGF-β ligand-receptor complex through clathrin-coated compartments versus caveolae can define the functional outcome of this cytokine (signal transduction versus degradation) (Guglielmo et al., 2003; Hoeller et al., 2005).

Beside smad-dependent pathway, activated TGF-β receptor complex can induce other smad-independent signaling pathways – including MAP kinases (Erk, JNK, and p38 MAPK), JUN, PI3K, PP2A, Rho, and PAR6 – that could either regulate smad signaling or result in other outcomes independent of smads (Derynck and Zhang, 2003; Feng and Derynck, 2005). Again, it is noteworthy that cellular context is an important determinant of the activation of such alternative pathways in response to TGF-β activation and receptor binding ((Derynck and Zhang, 2003; Feng and Derynck, 2005). Moreover, the *in vivo* significance of those non-smad signaling

factors in mediating TGF-β-related outcomes or regulation of smad-dependent signaling is still largely unknown.

Both smad-dependent and smad-independent signaling pathways initiated by TGF-β activation could potentially be involved in different steps of wound healing. Although the involvement of TGF-β in different stages of wound healing has been pointed out in the first part of this chapter, a specific summary of the roles, expression pattern, and consequences of TGF-β misregulation during wound healing will be given in the next section.

1.5.5. Wound healing and TGF-βs

Following wounding, the level of TGF-βs alters significantly (O'Kane and Ferguson, 1997). TGF-β isoforms are produced by macrophages, fibroblasts, keratinocytes, and platelets in different stages of wound healing (Roberts and Sporn, 1996). Studies on the expression pattern of three mammalian isoforms of TGF-β during wound healing have been mainly performed using cutaneous wounds, and have led to somewhat controversial results in different species. Those studies have examined TGF-β isoforms in different animal models including mice, rats, pigs, rabbits, sheep and humans (McMullen et al., 1995; O'Kane and Ferguson, 1997). Most of the immunohistochemistry and *in situ* hybridization studies in skin wound samples have shown a peak for TGF-β1 expression within 24 hours post-wounding that follows with a gradual decrease in the level of the cytokine and, in some studies, with a second peak at day 7 post-wounding (Kane et al., 1991; Frank et al. 1996; O'Kane and Ferguson, 1997). On the other hand, in Kopecki et al.'s (2007) study none of these peaks during day 1 or at day 7 was found; instead, they showed a peak expression of TGF-β1 at day 3 post-wounding in mice with a decline towards day 7. Finally, Nath et al. (1994) did not find a "second" peak but the "only" peak expression of TGF-β1 to occur at day 7 post-wounding in the rabbit model of incisional wounds. TGF-β1 was mostly detected in adult cutaneous wound healing in association with the ECM and within the fibroblasts and macrophages in the granulation tissue (Nath et al., 1994; McMullen et al., 1995). Migrating wound keratinocytes were either just weakly positive for TGF-β1 expression or completely negative (McMullen et al., 1995; Frank et al., 1996). However, TGF-β1 was detected in the epithelium adjacent to the wound edge and in the proliferated wound keratinocytes after wound closure (McMullen et al., 1995). TGF-β2 has been shown to follow

almost the same pattern as TGF-β1 during cutaneous wound healing (Nath et al., 1994; O'Kane and Ferguson, 1997). It was detected below the wound epithelium and in the granulation tissue (Frank et al., 1996). Most of the studies investigating the expression pattern of TGF-β3 have shown the peak expression of this isoform of TGF-β to be at or about day 7 post-wounding (Frank et al., 1996; O'Kane and Ferguson, 1997). Another study in rats has shown the upregulation of TGF-β3 as early as 1 hour post-wounding, which remained upregulated beyond 14 days (Ferguson and O'Kane, 1997). In all of these studies the upregulation of TGF-β3 has been associated with a downregulation of TGF-β1. TGF-β3 has been mostly detected in the wound epithelium (Frank et al., 1996). Its expression has also been found to some extent in the fibroblasts and other dermal cells of granulation tissue (McMullen et al., 1995).

The localization of TGF-βRI and TGF-βRII, as an indicator of TGF-β responsiveness during wound repair, showed a strong correlation with the TGF-β isoforms (Frank et al. 1996). As for the TGF-β isoforms, TGF-β receptors did not acculmulate in the migrating epidermis, but were localized to the epithelial layars after wound re-epithelialization (from day 7 to 10 post-wounding) (McMullen et al., 1995). However, according to McMullen et al., basal epithelium remained negative throughout the experiment. It has been shown that most of the fibroblasts in the granulation tissue were strongly positive for TGF-β receptors (Schmid et al., 1998). The peak expression of TGF-βRI and TGF-βRII has been found to occur between 1-5 days after peak expression of TGF-β isoforms (Gold et al., 1997). This may imply that although TGF-β ligands are present, they may not be completely functional until the optimal expression of TGF-β receptors. Yang et al. (1999) for the first time could analyze the level of active TGF-β in a short-term study of rat skin wound healing by TGF-β luciferase assay. They showed two peaks for the TGF-β activation that occurred 1 hour after injury and before day 7 post-wounding (Yang et al., 1999). Since TGF-β1 is the predominant isoform of TGF-β (>85%) in adult wounds (Hantash et al., 2008), it is conceivable to assume that this isoform is responsible for most of the detected TGF-β activity in Yang et al.'s study.

In summary, the peak expression of TGF-β1 at day 7 post-wounding, as shown by some of the above-mentioned studies, well correlates to the peak activity of TGF-β at about the same time (a small time difference observed between the two studies may reflect different

experimental settings). The peak expression and activity of TGF-β1 in addition to the expression of TGF-β receptors coincide with the peak expression of αvβ6 integrin that occurs around day 7 after cutaneous wounding (Haapasalmi et la., 1996). All of these data may suggest a potential for the αvβ6 integrin-mediated activation of TGF-β1 during wound healing. To better study such relationship, therefore, it seems plausible – and even necessary – to evaluate the expression of TGF-β isoforms in correlation with the expression pattern of αvβ6 integrin within a single experimental model in a long-term study.

TGF-β1 induces the recruitment of inflammatory cells, including macrophages, in addition to activating them (Clark, 1996). As mentioned earlier, TGF-β1 initiates granulation tissue formation through its pro-fibrotic effects for induction of ECM formation, promoting angiogenesis by upregulation of the angiogenic growth factor VEGF, and facilitating wound contraction through stimulation of the appearance of myofibroblasts (Roberts et al., 1986; Keski-Oja et al., 1988; O'Kane and Ferguson, 1997; Riedel et al., 2007; Meckmongkol et al., 2007). Although TGF-β1 influences keratinocytes in a few significant ways, its net effect on re-epithelialization remains controversial. Importantly, TGF-β1 induces re-distribution and neo-expression of keratinocyte integrins to facilitate their migration and residing in the wound provisional matrix (Margadant & Sonnenberg, 2010). It has also been shown that TGF-β1 promotes the migration of epithelial cells, *in vitro* (Koivisto et al., 1999, 2006). This could potentially have positive effect on the process of re-epithelialization. Other *in vitro* and *in vivo* studies, however, have shown that TGF-β1 inhibits keratinocyte proliferation suggesting an inhibitory role for this cytokine in re-epithelialization (Tucker et al., 1984; Moses, 1992; Amendt et al., 1998; Ashcroft et al., 1999). Genetic ablation of smad3 as TGF-β signaling mediator in mice has led to accelerated skin wound healing characterized with faster re-epithelialization rate, reduced infiltration of monocytes, and reduced deposition of ECM (Ashcroft et al., 1999). However, another study on TGF-β over-expressing mice showed that keratinocyte proliferation was slowed down in earlier stages of wound healing, but increased in later stages (Tredget et al., 2005). This shows the complexity of TGF-β signaling and function *in vivo* and the necessity for its tight local regulation. Finally TGF-β1 plays key roles in production, organization and sustaining of collagen fibers during tissue remodeling as the last stage of wound healing (Barrientos et al., 2008).

TGF-β2, in some aspects, functions similarly to TGF-β1 (O'Kane and Ferguson, 1997). It accelerates re-epithelialization and is involved in recruitment of inflammatory cells as well as fibroblasts to the wound site (Cox et al., 1992; Cordeiro et al., 1999). Moreover, TGF-β2 induces angiogenesis and participates in collagen synthesis and deposition (Roberts et al., 1986; Shah et al., 1995; Cordeiro et al., 1999).

TGF-β3, also known as the non-fibrogenic isoform of TGF-β, has been known to be involved in the recruitment of inflammatory cells and fibroblasts to the wound site. It is also a strong stimulant of angiogenesis (Merwin et al., 1991; Tyrone et al., 2000). TGF-β3 has been shown to preferentially promote epidermal cell migration but it decreases the migration of dermal cells (Bandyopadhyay et al., 2006). Unlike TGF-β1 and TGF-β2, TGF-β3 has been shown to result in a better organization of collagen fibers *in vivo* with a total effect of better healing and less scarring (Ferguson and O'Kane, 2004). There seems to be a cross regulation between the fibrogenic TGF-β1 and the non-fibrogenic TGF-β3, so that TGF-β1 peaks earlier and down-regulates earlier, while TGF-β3 appears later and stays longer during wound healing (Shah et al. 1995; Hsu et al. 2001; Ferguson and O'Kane 2004; Schrementi et al. 2008).

It has been shown that abnormal regulation of TGF-β isoforms relates to several human diseases, including fibrotic conditions and scar formation, and altering the expression pattern of these isoforms has been found to improve the healing process (Ferguson and O'Kane, 2004; Blobe et al., 2000). For example, continuous- or over-expression of TGF-β1 leads to aberrant wound conditions, but suppression of TGF-β1 (or more effectively TGF-β1 and TGF-β2) or topical application of TGF-β3 contributes to better healing and less scarring (Ferguson and O'Kane, 2004).

1.6. Objectives, rationale and hypothesis

In vitro studies have shown that αvβ6 integrin can activate TGF-β1 and TGF-β3, which play key roles in wound healing. The expression pattern and localization of αvβ6 integrin and its ligands, TGF-β1 and TGF-β3, has not been previously investigated in an extended time course of wound repair. In this study (Chapter 2), we have applied such approach to find out whether there is a potential for αvβ6 integrin-mediated regulation of fibrogenic TGF-β1 and anti-fibrogenic

TGF-β3 in experimental scarless gingival wounds of humans and in gingival and skin wounds of red Duroc pigs. **We hypothesize that the αvβ6 integrin spatio-temporally co-accumulates with fibrogenic TGF-β1 and anti-fibrogenic TGF-β3 in wound healing. The expression of TGF-β isoforms and their co-accumulation with the αvβ6 integrin is different in scarless gingival versus scar-forming skin wound.**

TGF-β1 is the predominant isoform of TGF-β which is abundantly deposited in the extracellular matrix of adult tissues and in the wound bed. Keratinocytes, therefore, not only are facing endogenously produced TGF-β1 during wound healing, but also are exposed to large amount of matrix-bound TGF-β1. In Chapter 3, we have applied cultured keratinocytes (which would resemble wound keratinocytes in many of their molecular and behavioral aspects) to investigate how αvβ6 integrin regulates relatively low levels of endogenously produced latent TGF-β1 versus high level of latent TGF-β1 bound to the extracellular matrix (ECM). **Our hypothesis is that the αvβ6 integrin expressed in wound keratinocytes interacts with TGF-β1 and has a dual role in activating TGF-β1 signaling pathway and reducing TGF-β1 levels by endocytosis or other mechanisms to protect the cells from inhibitory effects of TGF-β1 on cell proliferation.**

1.7. Significance

Scars from burn wounds or traumatic injuries in skin may result in disfiguring and debilitating consequences. Gingival wounds, on the other hand, have been shown to heal with no or minimal scarring. Comparing the molecular and biological aspects of scar-free gingival to scar-forming skin wounds could provide valuable information about the mechanisms why wounds in the adult skin produce scar. Considering the involvement of αvβ6-mediated activation of TGF-β1 in fibrotic conditions, such as pulmonary, renal, and hepato-biliary fibrosis, it is conceivable to speculate that similar mechanisms are also involved in scar formation in skin. Therapeutic targeting of a locally expressed molecule such as αvβ6 integrin instead of a multi-functional growth factor such as TGF-β1 is much preferred in preventing or managing TGF-β1-mediated pathological conditions, including scarring. Investigating a potential for αvβ6 integrin-mediated regulation of TGF-β isoforms during wound healing, as well as studying the nature of

such regulation in wound keratinocytes is critical for development of novel therapeutic modalities to target αvβ6 integrin in the course of wound healing to minimize scar formation.

1.8. References

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Chapter 2: Expression of Integrin αvβ6 and TGF-β in Scarless vs. Scar-forming Wound Healing[1]

2.1. Introduction

Wound healing is a complex process that restores tissue integrity. It is composed of coordinated functions and interactions of different cell types, extracellular matrix (ECM), cytokines and growth factors. Adult cutaneous healing results in accumulation of collagen-rich scar tissue that is slowly remodeled over time (Singer et al., 1999; Midwood et al., 2004). Dysregulation of skin wound healing following burn wounds, traumatic injuries or certain surgical procedures can result in serious wound complications, such as hypertrophic scars (Clark, 1996; Ghahary et al., 1993). Interestingly, however, oral mucosal wounds show accelerated healing with no or minimal scarring as compared to skin (Häkkinen et al., 2000a; Szpaderska et al., 2003). The mechanisms underlying scarless oral wound healing have not been fully investigated. However, the absence of scar in oral mucosal wounds may in part be due to the presence of saliva and specific microflora in the oral cavity or the fetal-like phenotype of the cells in the oral mucosa (Sciubba et al., 1978; Schor et al., 1996; Häkkinen et al., 2000a; Szpaderska et al., 2003; Schrementi et al., 2007). Therefore, comparing the biological mechanisms regulating scarless oral mucosal versus scar-forming dermal wound healing could provide valuable information about the pathophysiology of scar formation.

Transforming growth factor β (TGF-β) is a multi-functional growth factor with several critical roles during normal wound healing. TGF-β regulates wound re-epithelialization, inflammation and promotes connective tissue regeneration (Verrecchia et al., 2002). On the other hand, dysregulation of TGF-β plays a key role in abnormal wound healing, including scar formation. There are three mammalian isoforms; TGF-β1, -β2 and -β3 have distinct roles in wound repair. TGF-β1 over-activity is considered the primary pro-fibrotic factor that causes excessive ECM accumulation and scar formation and suppression of its activity by pharmacological or genetic approaches results in markedly reduced scarring (McCallion and

[1]A version of this manuscript has been published. Expression of integrin alphavbeta6 and TGF-beta in scarless vs scar-forming wound healing. Eslami, A., Gallant-Behm, C.L., Hart, D.A., Wiebe, C., Honardoust, D., Gardner, H., Häkkinen, L., Larjava, H.S. *J Histochem Cytochem*. 2009 Jun; 57(6):543-57.

Ferguson, 1996; O'Kane and Ferguson, 1997; Shah et al., 2000). On the other hand, exogenous application of the anti-fibrogenic TGF-β3 or intrinsic abundance of TGF-β3 relative to TGF-β1 has been associated with improved wound healing outcomes (Shah et al., 1995; Hsu et al., 2001; Ferguson and O'Kane, 2004; Schrementi et al., 2007). TGF-β isoforms are synthesized as latent molecules, consisting of mature TGF-β that is covalently bound to the latency-associated peptide (LAP). This latent complex associates with a family member of the latent TGF-β binding proteins (LTBPs) that facilitates TGF-β storage in the ECM. To be functional, TGF-β must be activated. There are several activators of TGF-β that can dissociate the mature TGF-β from LAP allowing it to interact with its cell surface signaling receptors (Annes et al., 2003). Integrin-mediated activation appears to be the main mechanism of TGF-β activation in vivo (Yang et al., 2007). Integrin αvβ6 can activate both fibrogenic TGF-β1 and anti-fibrogenic TGF-β3 *in vitro* through a mechanism that requires LTBP-1 (Annes et al., 2002; Annes et al., 2004; Keski-Oja et al., 2004). The β6 integrin-knockout mouse share similarities in phenotype with the TGF-β1-knockout animals, including exaggerated lung and skin inflammation suggesting a role for αvβ6 integrin in the activation of TGF-β1 in vivo (Huang et al., 1996). Moreover, the β6-knockout mice are protected from TGF-β1-mediated lung fibrosis induced by bleomycin (Munger et al., 1999). Furthermore, TGF-β1 activation via αvβ6 integrin contributes to renal and radiation-induced fibrosis (Hahm et al., 2007; Puthawala et al., 2008).

Integrin αvβ6 is an epithelial cell surface receptor that is not normally present in adult tissue but its expression is strongly induced in keratinocytes during wound healing (Breuss et al., 1993; Breuss et al, 1995; Häkkinen et al., 2000b). We have previously shown that the expression of αvβ6 integrin is induced at the wound basal epithelium of both human gingival and skin wounds (Haapasalmi et al., 1996; Häkkinen et al., 2000b). However, the function of αvβ6 integrin during wound healing, especially in regards to the regulation of TGF-β1 and TGF-β3 activity, remains largely unclear. In order to test the hypothesis that αvβ6 integrin may interact with TGF-β in the wound epithelium, we studied the spatio-temporal colocalization of these and other key molecules involved in TGF-β activation in the human oral mucosal wound epithelium. In addition, we studied the activity of the TGF-β pathway in the early stages of wound healing by gene expression profiling. We also hypothesized that the expression and localization of αvβ6

integrin and TGF-β1 and TGF-β3 are different in scarless gingival wounds and scar-forming skin wounds. To this end, we compared expression and localization of these molecules in skin and oral mucosal wounds over time using a well-established pig model.

2.2. Materials and methods

2.2.1. Tissue samples

Human wounds - The experimental protocol for human wounding was approved by the Research Ethics Board of the University of British Columbia and complies with the ethical rules for human experimentation that are stated in the 1975 Declaration of Helsinki. Full-thickness excisional wounds (2 x 12 mm) were created in the gingiva of four healthy volunteers (males and females, 22-35 years of age) at least 3 mm away from the teeth margins. The wounds were left uncovered to heal for up to 60 days. Three biopsies (4 mm diameter) from different individuals were collected per time point**.** The tissue harvested from the initial wound (unwounded tissue) and the wound biopsies were rinsed in phosphate buffered saline (PBS) and either placed in cryotubes or embedded in Tissue-Tek Optimal Cutting Temperature compound (OCT; Sakura Finetek Inc., Torrance, CA) and snap frozen in liquid nitrogen and stored at -86°C for later use. Transcriptional profiling of day-1, -3 and -7 wound samples was compared to their individual 0-day (non-wounded) sample using Affymetrix protocols (Affymetrix Inc., Santa Clara, CA). Tissue samples embedded in Tissue-Tek O.C.T. were cut to 6 µm frozen sections with a 2800 Frigocut Cryostat Microtome (Leica, Nussloch, Germany), and transferred to 3-aminopropyltriethoxysillane-coated slides, fixed with cold acetone, air-dried and kept at -86°C until use.

Pig wounds - The experimental protocol was performed in accordance with Canadian Council on Animal Care guidelines and with a protocol approved by the Animal Care Committee of the Faculty of Medicine at the University of Calgary. Six juvenile (20-25kg) female red Duroc pigs were obtained from the Neufeld farm (Acme, AB) and were housed at the University of Calgary Life Sciences Research Station. Six, identically sized wounds as created in human gingiva were made in both the palatal gingiva and dorsal skin of each animal. Briefly, the animals were pre-medicated with intramuscular ketamine (15 mg/Kg) and acepromazine (0.4 mg/Kg), and general anesthesia was induced by the administration of 1-2% isofluorane by mask.

The gingival wounds were created as described above for the human wounds. Identical size full-thickness skin wounds were created on the dorsal skin as described previously (Gallant et al., 2004). A fentanyl transdermal patch (75 mg/hour) was used for long-term pain control (72 hours), and butorphanol (0.1 mg/kg, IM) was administered for break-through pain for the first 8 hours. Tissue biopsies (4 mm in diameter) harvested from gingiva and skin of red Duroc pigs were collected from unwounded tissue (day 0 samples) and from the wounds 1, 3, 7, 14, 21, 35 and 49 days post-wounding (N=6 pigs per time point for skin; N=3 pigs per time point for gingiva). The tissue biopsies were then snap frozen and used for either RNA extraction or frozen sectioning (see above).

2.2.2. Evaluation of wound healing

Clinical evaluation of the wounds in various time points was performed for the signs of scar formation based on clinical scar assessment scale by Wong et al. (manuscript in preparation) and recorded by taking standardized photographs using a digital camera. In addition, histological evaluation of the wounds was performed based on the histological scar assessment scale (Wong et al., submitted) of hematoxylin and eosin stained wound sections. The results of scar assessment scales are shown elsewhere (Wong et al., manuscript in preparation) and only representative clinical and histological images from the last time point studied (49 days post-wounding) are presented here.

2.2.3. DNA microarray

DNA micro-array was performed using the Affymetrix standard protocol (Affymetrix User's Guide). Total RNA was extracted from seven unwounded (day 0) and three wounded human gingival tissue samples per time point (day 1, 3, and 7) using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA) followed by a clean up process using RNeasy kits (QIAGEN, Valencia, CA) according to the manufacturer's protocols. Poly(A) mRNA was reverse transcribed to generate double stranded cDNA using a 24-mer oligodeoxythymidylic acid primer with a T7 RNA polymerase prNSomoter site added to the 3′ end T7-oligo (dT) (Superscript cDNA Synthesis System; Life Technologies, Rockville, MD; Dumur et al., 2004). The cDNA was used as a template for *in vitro* transcription (IVT) to yield biotin-labeled cRNA. In order to obtain gene expression profiles, the biotinylated cRNA was heat-fragmented and then

hybridized to oligonucleotide GeneChips (Affymetrix, U95Av2) which contained different probe sets representing different genes. After hybridization (16 h, 45°C), the probe arrays were washed in non-stringent buffer and stained with streptavidin phycoerythrin followed by an antibody amplification step. Arrays were scanned for fluorescence intensities using Affymetrix scanner. Thereafter, the scanned image was analyzed with Affymetrix Micro-array Suite 5.0. Results are presented as the fold changes between pooled control samples (unwounded gingiva) and pooled test samples (1-, 3-, and 7-day-old wounds). Genes whose expression did not exceed the background level were omitted. Analysis of variance (ANOVA) was used to determine the significantly upregulated and downregulated genes for all patients at each wound time point (p-value ≤ 0.01). In this paper, the genes that are related to TGF-β activation or signaling are presented.

2.2.4. Histology and immunohistochemistry

Frozen sections from different time points of human and pig wounds were thawed at room temperature before fixation in acetone (-20°C) for 5 minutes. Thereafter, they were stained with hematoxylin and eosin, mounted using Entellan (Merck, Whitehouse Station, NJ), and photographed using a digital camera (Nikon Coolpix 995, Tokyo, Japan) attached to an Axiolab E light microscope (Carl Zeiss, Jena, Germany). For immuno-localization studies using single or double immunofluorescence staining the following antibodies were used: αv (monoclonal antibody MAb L230; purified in our laboratory; Houghton et al., 1982), β6 (β6-B1; Huang et al., 1996), TGF-β1 (Promega, Madison, WI), TGF-β3 (PAb; Santa Cruz Biotechnology), LTBP-1 (Pharmingen, SanDiego, CA), CTGF (Abcam, Cambridge, UK), procollagen type-I (Chemicon International, Temecula, CA), MMP-9 (Chemicon International), TSP-1 (Medi Corp, Montreal, QC, Canada). For immunofluorescence staining, sections were first incubated with PBS containing bovine serum albumin (BSA, 10 mg/ml) and Triton X-100 (0.1%) at room temperature for 30 minutes. The tissue sections were then incubated with a primary antibody in PBS containing BSA (1 mg/ml) and Triton X-100 (0.01%) at 4°C overnight. Thereafter, for double immunofluorescence staining, the sections were washed and incubated with a second primary antibody at room temperature for 1 h. After washings, the samples from both single and double immunofluorescence stainings were incubated with appropriate Alexa-conjugated secondary antibodies (Alexa 488 and Alexa 546; Molecular Probes Inc., Eugene, OR) against

primary antibodies at room temperature in the dark for 1 h. The control samples were exposed to either secondary antibodies only or non-immune IgG and gave negligible immunoreactivity (not shown). Subsequently, the slides were mounted using Immuno-Mount solution (Thermo Shadon, Pittsburgh, PA), examined by a Zeiss Laser Confocal Scanning Microscope 10 (LSM 10; Carl Zeiss), and images were captured using Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada).

2.2.5. Extraction of RNA from pig skin and gingival

Triplicate frozen tissue samples from pig gingiva and skin (unwounded tissue and different time points of the wounds) were placed on dry ice, cut into small pieces using a razor blade, and transferred into the lysis buffer provided in the applied RNA extraction kit (Qiagen RNeasy Fibrous Tissue Mini Kit, Qiagen Inc., Mississauga, ON, Canada). The samples were homogenized using hard-tissue Omni-tip^{TM} plastic probes (Omni International, Marietta, GA) attached to a rotor stator (Power Gen 1000, Fisher Scientific, Ottawa, ON, Canada). To eliminate the cross-contamination between the samples, the plastic probes were washed in RNase-free water three times with the rotor stator operating. Subsequently, RNA was extracted from homogenized samples according to the manufacturer's protocol (Qiagen RNeasy Fibrous Tissue Mini Kit). The concentration of RNA in each sample was determined by spectrophotometry (GeneQuant, Biochrom LTD, Cambridge, England) at 260 nm wavelength. The RNA samples were considered acceptable if the 260:280 nm was greater than 1.8. Extracted RNA was stored at -80°C for later use.

2.2.6. Polymerase chain reaction

The synthesis of cDNA was performed by reverse transcription of RNA (1 µg) using iScript^{TM} Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's standard protocol. Amplification of β6 integrin, TGF-β1, TGF-β3, and β-actin (as housekeeping gene) cDNA was performed using the MiniOpticon Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Primer sequences used for cDNA amplification were: 5-ATA GTT CCA GCA TCG TTC AG-3 (forward), and 5-CGA ACT TGA ACT TGC AGA G-3 (reverse) for β6 integrin; 5-TTT CGC CTC AGT GCC CA-3 (forward), and 5-GCCAGAATTGAACCCGTTAA-3 (reverse) for TGF-β1; 5-GGA CAC CAA TTA CTG CTT

CC-3 (forward), and 5-CCA GAT CCT GTC GGA AGT C-3 (reverse) for TGF-β3; and 5-CTG TGG CAT CCA CGA AAC-3 (forward), and 5-CAG ACA GCA CTG TGT TGG-3 (reverse) for β-actin. The β6, TGF-β1, and TGF-β3 levels were normalized to β-actin mRNA levels using the comparative C_T method and reported as ratio of gingival to skin values in unwounded samples and different wound time points. Melting curve analyses were performed for all amplifications to verify that only single products were generated from the reactions. The gene expression levels of β6 integrin, TGF-β1, and TGF-β3 were reported from day 0 to day 49 in both gingival and skin wounds of red Duroc pigs. Moreover, the ratio of the gene expression in each time point in gingiva relative to skin is presented. Each set of PCR was repeated at least three times to check the reproducibility of the data. The differences between the gene expression levels in all wound time points were compared to unwounded samples using Student's t test. Analysis was performed using Microsoft Excel software and a p value ≤ 0.05 was considered statistically significant.

2.3. Results

2.3.1. Transcriptional profiling of gene expression in scarless human gingival wounds depicts high remodeling and matrix production activity and involvement of molecules in the TGF-β pathway

Molecular events in the early wound healing process may dictate whether a wound forms a scar or not (Ferguson and O'Kane, 2004). Therefore, we performed transcriptional profiling using RNA isolated from the whole tissue biopsies obtained from unwounded human gingival tissue and from early gingival wounds (1-, 3-, and 7-days post-wounding). The known genes regulated by TGF-β or partners of the TGF-β signaling pathway were selected from the micro-array data for closer analysis (Table 2.1). Interestingly, the results did not reveal any changes in the expression levels of TGF-β1 or TGF-β receptor I at any time points relative to unwounded tissue; likely because of low transcript levels of these genes. However, TGF-β receptor II (TGF-βRII) and TGF-βRIII were initially moderately down-regulated in 1-day-old wounds and then remained unchanged compared to unwounded tissue (Table 2.1). On the other hand, the expression of integrin αv and anti-fibrogenic TGF-β3 as well as LTBP-1 were upregulated in 7-day-old wounds (Table 2.1).

Table 2.1. Transcriptional profiling of TGF-β–related genes at different time points of human gingival wounds (1, 3, and 7 days old) compared with unwounded tissue (day 0). Results are presented as the fold changes between pooled samples (n53; each wound sample was compared with its individual day 0 sample) analyzed by ANOVA to determine the significant changes over unwounded tissue (p,0.01). TGF, transforming growth factor; NC, no change; CTGF, connective tissue growth factor; ECM, extracellular matrix.

Molecule	Day-1	Day-3	Day-7
TGF-β signaling and activation molecules			
Transforming growth factor β1 (TGF-β1)	NC	NC	NC
Transforming growth factor β receptor I (TGF-βRI)	NC	NC	NC
Transforming growth factor β receptor II (TGF-βRII)	-1.60	NC	NC
Transforming growth factor β receptor III (betaglycan, TGF-βRIII)	-2.31	NC	NC
Transforming growth factor β3 (TGF-β3)	NC	NC	2.60
Latent transforming growth factor β binding protein-1 (LTBP-1)	NC	NC	1.85
Integrin αv (ITGαv)	NC	NC	1.82
Connective tissue growth factor (CTGF)	2.71	4.15	4.74
ECM molecules			
Collagen type-I, α2 (CoL1α2)	-21.13	-1.84	4.22
Collagen type-III, α1 (Col3α1)	-6.01	NC	2.50
Fibronectin-1 (FN-1)	-2.51	NC	12.67
Tissue Remodeling Molecules			
Matrix metalloproteinase-1 (MMP-1)	40.11	21.72	17.07
Matrix metalloproteinase-9 (MMP-9)	16.71	NC	14.48

In addition, the expression of connective tissue growth factor (CTGF), which is one of the targets of TGF-β signaling and mediates many of biological effects of TGF-β (Perbal, 2004), was highly upregulated already in 1-day-old wounds and remained high also in 3- and 7-day-old wounds (Table 2.1). The expression of extracellular matrix molecules that can be induced by TGF-β (Varga et al., 1987), including type I and III collagens and fibronectin was strongly down-regulated in 1-day-old wounds and then up-regulated in 7-day-old wounds. The expression of matrix metalloproteinase-1 (MMP-1) and MMP-9, expression of which can be regulated by TGF-β and which are also able to activate TGF-β (Salo et al., 1994; Yu and Stemenkovic 2000; Mu et al., 2002), was strongly increased at 1 and 7 days post-wounding (Table 2.1). Taken together, we found that the gene expression level of αv integrin along with TGF-β3 and LTBP-1 and TGF-β-regulated genes (type I and III collagens, fibronectin, CTGF, MMP-1 and MMP-9) were upregulated in 7-day-old human gingival wounds.

2.3.2. TGF-β1 and TGF-β3 co-localize with αvβ6 integrin in 7-day-old gingival wounds

To validate the micro-array gene expression data and to find out which cells express these molecules, the localization and relative staining intensity level of selected proteins in unwounded gingiva and 7-day-old human gingival wounds was investigated. TGF-β1 and TGF-β3 were almost undetectable in the unwounded epithelium or connective tissue (Figure 2.1.A). However, in 7-day–old wounds, TGF-β1 staining was very intense and localized at the basal aspect of wound epithelium. TGF-β3 was also localized abundantly to the basal aspect of wound keratinocytes but was also present in the suprabasal cell layers. Expression of both TGF-β1 and –β3 was weak in the granulation tissue. The gene array data showed upregulation of the αv integrin subunit expression in the 7-day-old wounds. Accordingly, there was strongly increased immunostaining of this integrin subunit in the 7-day-old wound epithelium as compared to unwounded tissue (Figure 2.1.A). Our previous observations have also demonstrated high expression of αv integrin subunit in 7-day-old human gingival wounds and have shown that in this location the αv subunit forms heterodimers with the β6 integrin and probably also with the β1 subunit (Haapasalmi et al., 1996; Koivisto et al., 1999; Häkkinen et al., 2000b; Larjava et al., 2002). Our immunostaining confirmed colocalization of the αv and β6 integrins in the basal

epithelium of the 7-day-old wounds (Figure 2.1.A). Integrin αv was also localized into wound granulation tissue (Figure 2.1. A).

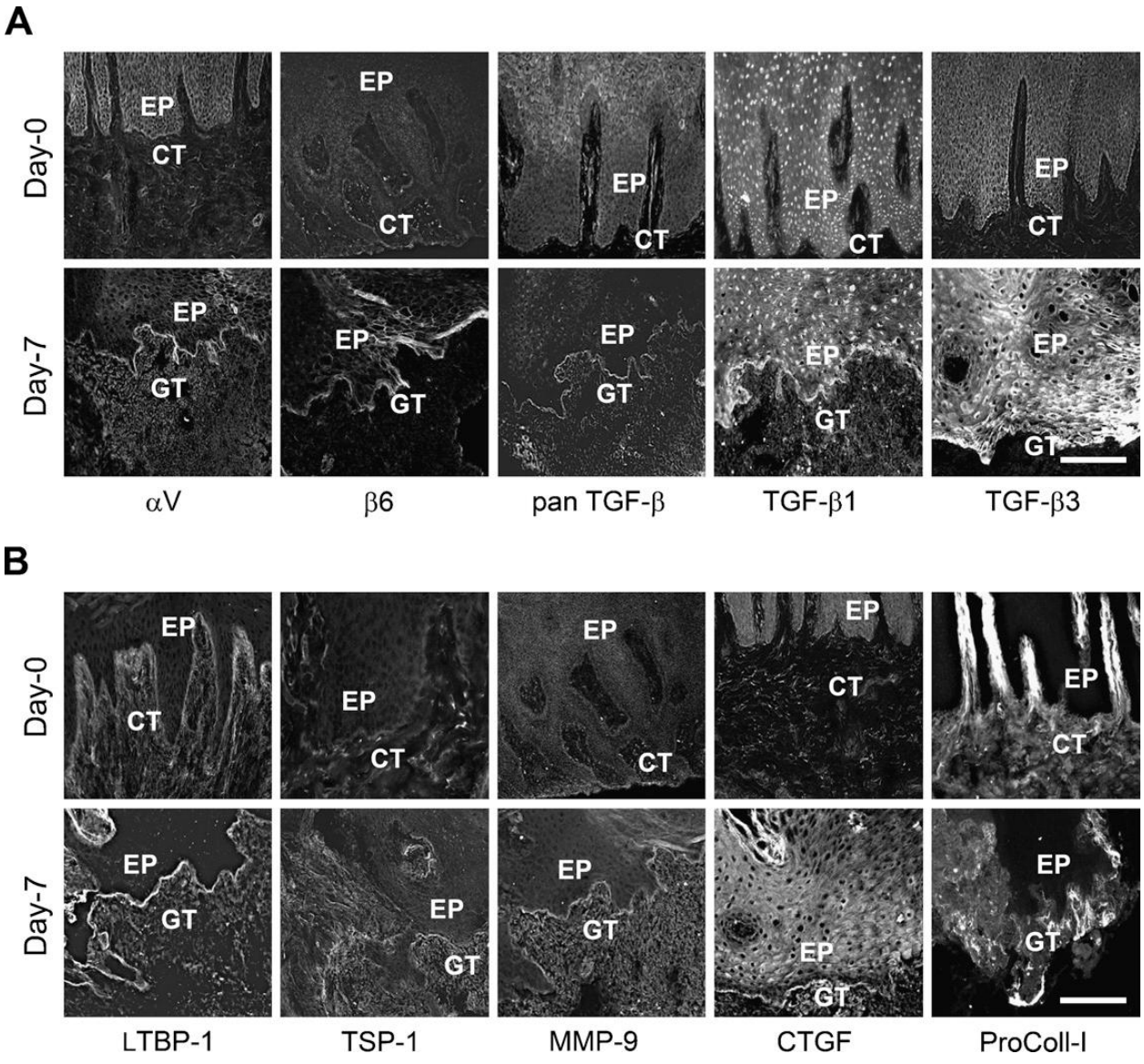

Figure 2.1. Immunostaining of representative samples from unwounded human gingival tissue (day 0) and 7-day-old human gingival wound. Higher staining intensity of αv, β6, pan TGF-β (TGF-β1, 2, 3), TGF-β1, TGF-β3 (A), and LTBP-1, TSP-1, MMP-9, CTGF and pro-collagen-I and pro-collagen-I (B) was found in the 7-day-old human gingival wound basal epithelium compared with unwounded gingiva. EP, epithelium; CT, connective tissue; GT, granulation tissue. Magnification bar = 200 μm.

Next, we examined the localization of LTBP-1, the TGF-β-binding protein that is necessary for αvβ6 integrin–mediated activation of TGF-β, and MMP-9 (that can activate TGF-β) which showed upregulation in the 7-day-old wounds in our gene array analysis (Figure 2.1.B). In normal tissue, LTBP-1 was localized in the gingival connective tissue with the most distinct staining at the epithelial basement membrane zone (BMZ). In 7-day-old wounds, the intensity of the LTBP-1 staining was increased, especially at the wound epithelium BMZ (Figure 2.1.B). The immunoreactivity of MMP-9 was almost negligible in the unwounded tissue but showed strongly increased immunostaining in the granulation tissue and BMZ of the wound epithelium of 7-day-old wounds (Figure 2.1.B). Next, we evaluated the accumulation and localization of other potential non-integrin activators of TGF-β in 7-day-old wounds. Expression of TSP-1 was almost negligible in the normal tissue. In the wounds, TSP-1 was localized diffusely throughout the granulation tissue with no clear preference to the BMZ (Figure 2.1.B). We then examined CTGF and type I procollagen, two molecules that can be induced by TGF-β and which showed strongly increased expression in the 7-day-old wounds. CTGF was localized in cells, likely fibroblasts, of the gingival connective tissue of unwounded tissue. In 7-day-old wounds, CTGF was present in the granulation tissue, BMZ and suprabasal cell layers of the wound epithelium (Figure 2.1.B). Type I procollagen was expressed around the blood vessels in the connective tissue papillae of the normal tissue (Figure 2.1.B). In the wounds, expression of type I procollagen was observed in the granulation tissue, especially immediately underneath the fused wound epithelium (Figure 2.1.B).

To assess whether it is possible that αvβ6 integrin and TGF-β interact in the wound epithelium we performed double immunofluorescence staining for these molecules in the 7-day-old wounds (Figure 2.2). TGF-β1 colocalized with αvβ6 integrin at the BMZ (Figure 2.2). TGF-β3 also co-localized with αvβ6 integrin around the basal keratinocytes of the fused wound epithelium (Figure 2.2). In summary, we observed that αvβ6 integrin along with the key molecules involved in αvβ6 integrin-mediated activation of TGF-β and certain downstream targets of TGF-β signaling were coordinately upregulated and spatio-temporally colocalized in 7-day-old human gingival wounds.

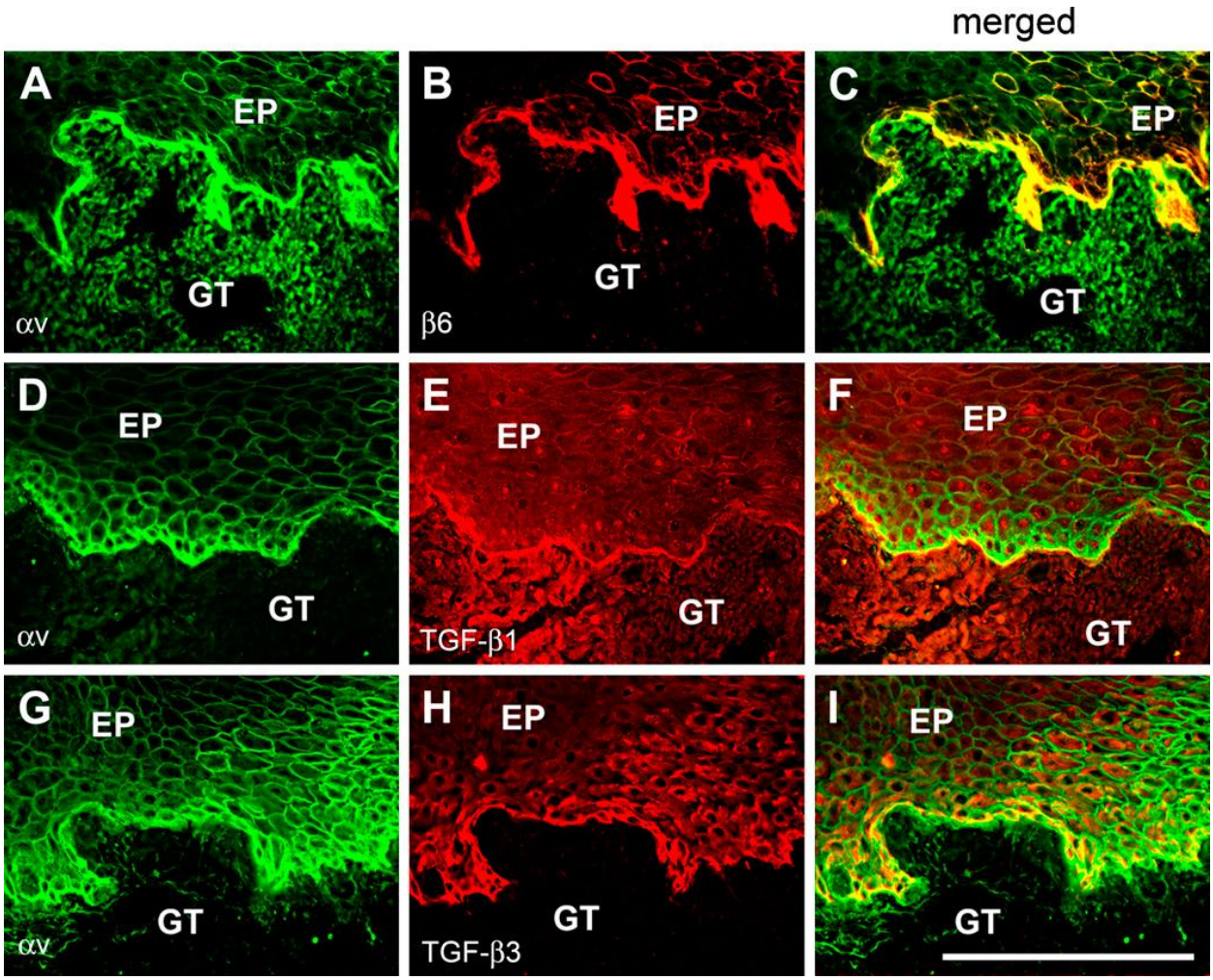

Figure 2.2. The colocalization of αv integrin with its binding partner β6 (A-C), TGF-β1 (D-F), and TGF-β3 (G-I) at the wound basal epithelium. Immunostaining of a representative sample from a 7-day-old human gingival wound is shown. Note that colocalization of different molecules is shown by yellow color in the merged images. EP, epithelium; GT, granulation tissue. Magnification bar = 200 μm.

2.3.3. Localization of αvβ6 integrin, TGF-β1 and TGF-β3 in scarless human gingival wounds up to 60 days post-wounding

Having established that αvβ6 integrin is colocalized with two isoforms of TGF-β, suggesting that it may participate in TGF-β activation, we investigated the localization of these proteins at later stages of gingival wound healing. Human gingival wounds showed minimal scarring at both clinical and histological levels after 60 days of healing (Figure 2.3; Wong et al., manuscript in preparation).

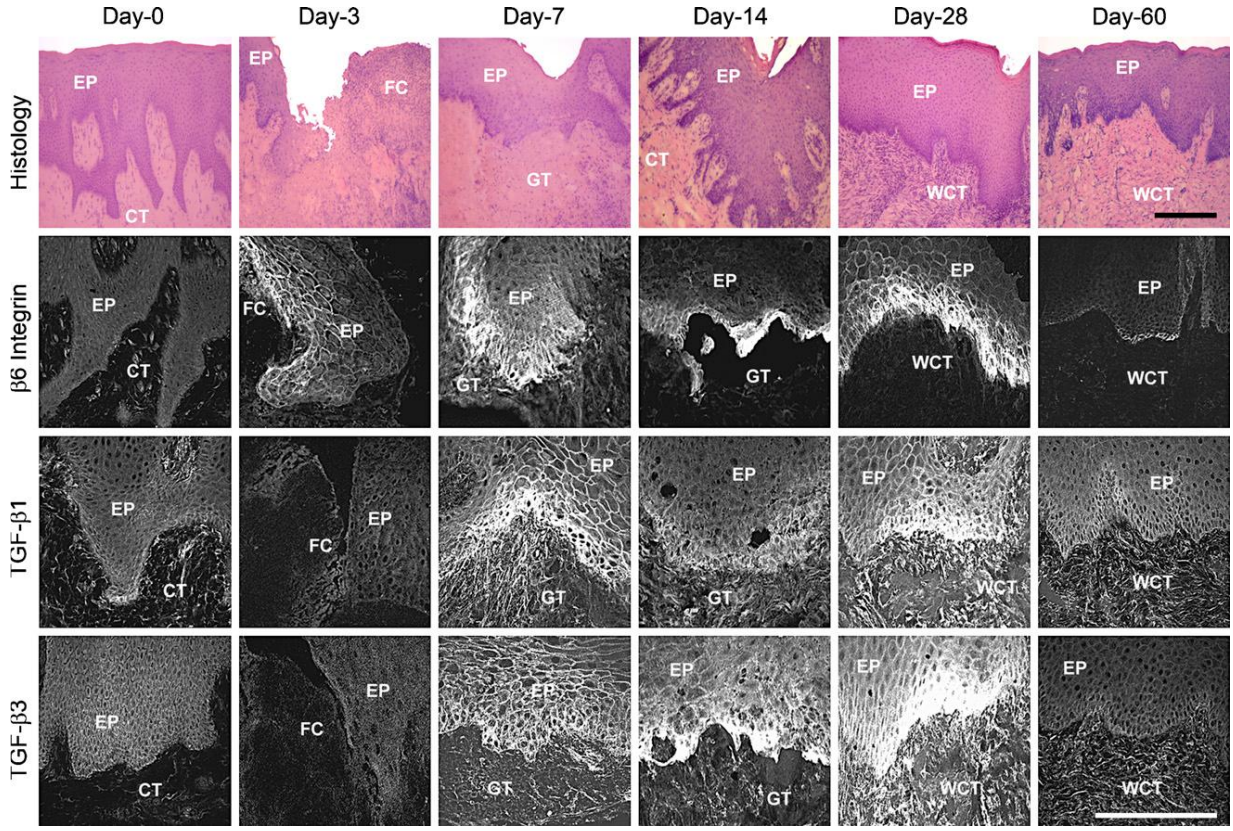

Figure 2.3. Immunolocalization of β6 integrin, TGF-β1 and TGF-β3 in 0- to 60-day-old human gingival wounds. Representative histological images were taken from hematoxylin and eosin stainings of the nonwounded and wounded samples. EP, epithelium; CT, connective tissue; FC, fibrin clot; GT, granulation tissue; WCT, wound connective tissue. Magnification bar = 200 μm.

Immunoreactivity for β6 integrin was first detected in the basal and suprabasal cell layers of the migrating gingival epithelium of 3-day-old wounds (Figure 2.3). Staining intensity of β6 integrin increased in the basal keratinocytes of the fused wound epithelium in the 7-day-old wounds and remained high until day 28 and then declined but was still detectable in the 60-day-old wounds (Figure 2.3). Immunostaining intensity of TGF-β1 was increased in the wound keratinocytes and granulation tissue in the 7-day-old wounds and remained high in wound keratinocytes up to 28 days, and then declined to unwounded tissue level by day 60 (Figure 2.3). The staining pattern of TGF-β3 followed that of TGF-β1 and was mainly confined to the basal keratinocytes of the wound area (Figure 2.3). Taken together, αvβ6 integrin is spatio-temporally coordinately colocalized with both of its ligands, TGF-β1 and -β3 during scarless gingival wound healing.

2.3.4. Comparison of wound healing in the gingiva and skin of red Duroc pigs

To determine whether the expression of αvβ6 integrin and TGF-βs show differential distribution in scar-forming wound healing, we chose the red Duroc pig model, a model of hypercontracted, hypertrophic-like healing (Gallant et al., 2004). We have demonstrated that gingival wounds in Red Duroc pigs heal similarly to human gingiva with minimal scarring (Wong et al., manuscript in preparation). However, even small dermal wounds produce scarring similar to human skin (Zhu et al., 2007). Consistent with our previous findings, 49-day-old gingival wounds showed minimal scarring both at the clinical and histological levels (Figure 2.4). Dermal healing, however, produced clinically visible scarring and histological changes were consistent with scar formation and showed areas with hypercellularity and disorganized collagen fibers (Figure 2.4).

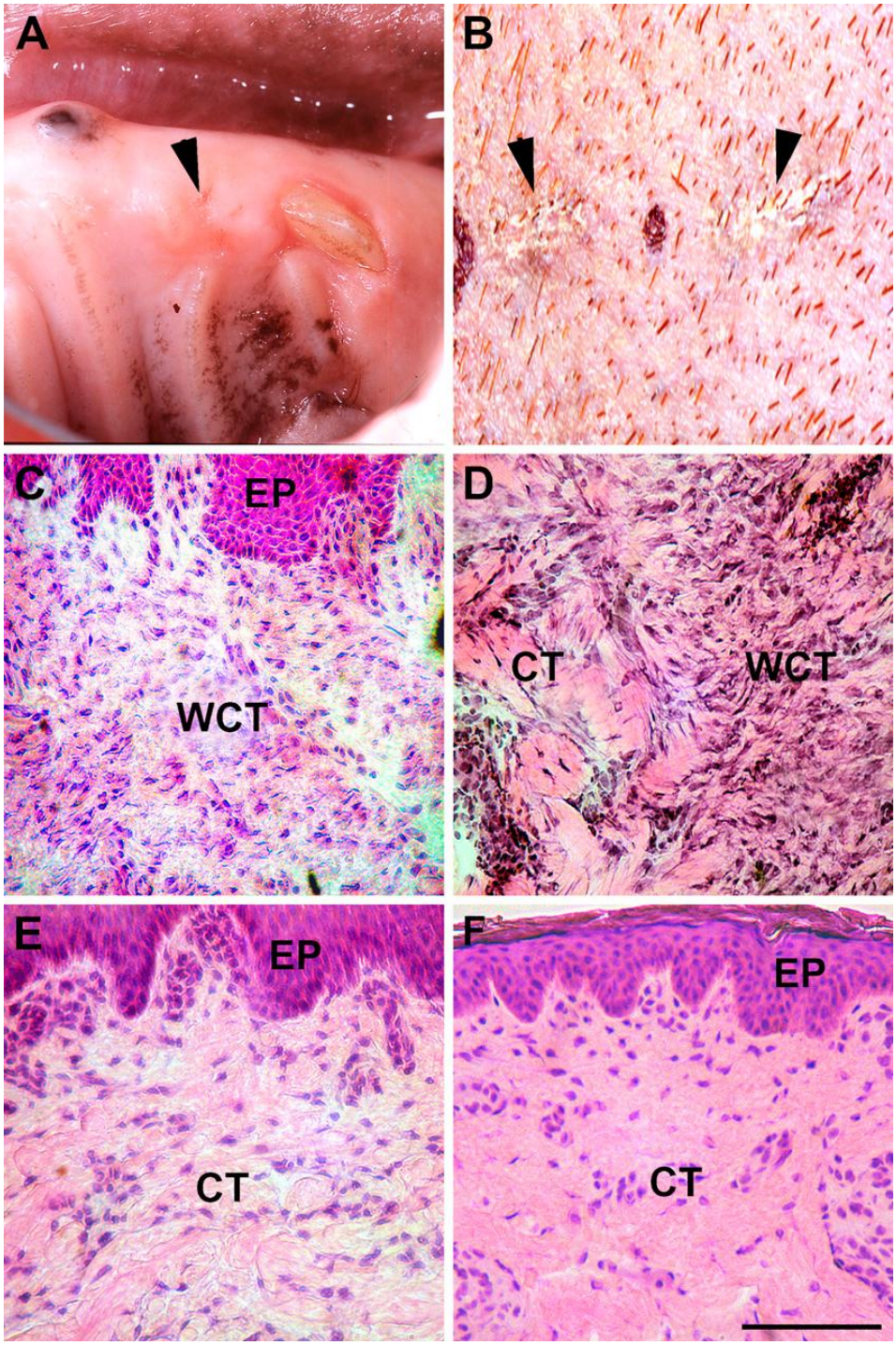

Figure 2.4. Representative clinical and histological (hematoxylin-eosin staining) images of 49-day-old gingival (A, C) and skin (B, D) wounds and of unwounded gingiva (E) and skin (F) of red Duroc pigs. Arrow heads indicate the healing wound site in the gingiva (A) and scar in the skin (B). EP, epithelium; WCT, wound connective tissue; CT, connective tissue. Magnification bar = 200 µm.

2.3.5. Localization of αvβ6 integrin, TGF-β1, and TGF-β3 in scarless versus scar-forming wounds of red Duroc pigs

Immunostaining showed that integrin β6 protein was not detectable in unwounded gingiva and skin of red Duroc pigs (day 0). However, β6 integrin immunostaining was strongly increased in the basal wound keratinocytes of the fused epithelium of 7- and 14-day-old gingival and skin wounds (Figure 2.5).

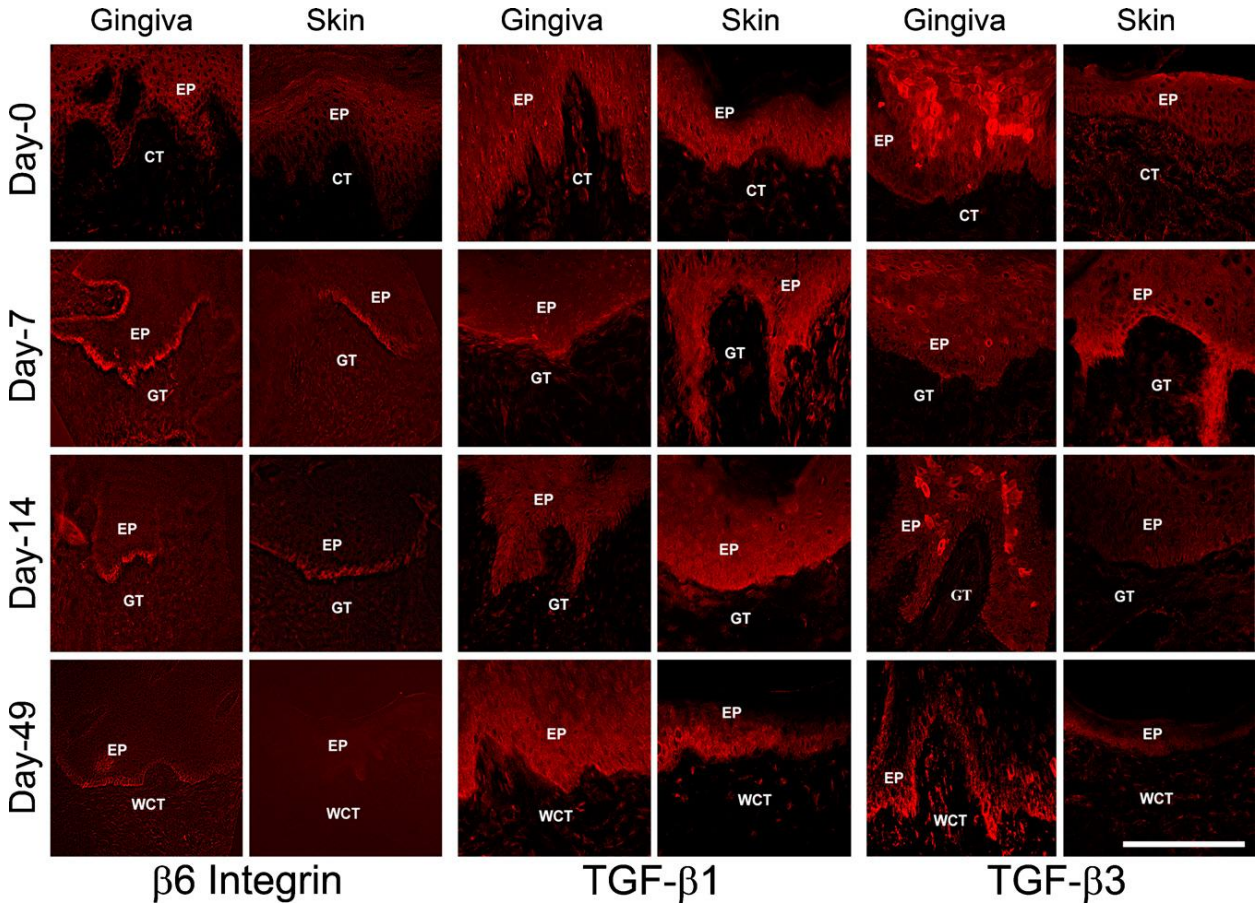

Figure 2.5. Representative images of immunolocalization of β6 integrin, TGF-β1 and TGF-β3 in 0- to 49-day-old gingival and skin wounds in red Duroc pigs. EP, epithelium; CT, connective tissue; GT, granulation tissue; WCT, wound connective tissue. Magnification bar = 200 μm.

In the 49-day-old wounds, β6 integrin was still detectable in the basal keratinocytes of the wounded gingiva but not in the skin (Figure 2.5). Species specificity of the TGF-β antibodies to pig specimens was tested in Western blotting of the frozen sections of 7-day-old wounds. Specific band corresponding to the latent TGF-β complex was detected with both TGF-β1 and-β3 antibodies, indicating that these antibodies were reactive to pig TGF-βs (data not shown). Immunostaining for TGF-β1 was detected with the same intensity in nonwounded tissue and in all wounds in the gingival and skin wound basal epithelium and some connective tissue cells (Figure 2.5). Immunostaining for TGF-β3 was mostly confined in the supra-basal keratinocytes of gingival epithelium of the normal gingiva and 7-day and 14-day-old gingival wounds (Figure 2.5). In the 49-day-old wounds, TGF-β3 was predominantly localized to the gingival wound basal epithelium and some connective tissue cells (Figure 2.5). In the skin, however, TGF-β3 was only detected on day 7 at the wound basal keratinocytes and in association with some stromal cells in unwounded tissue and in 49-day-old wounds (Figure 2.5). In summary, in red Duroc pig wounds β6 integrin was detected in the wound epithelium at all time points in the gingiva but its accumulation was downregulated by day 49 post-wounding in the skin. TGF-β3 localized to the basal epithelium in the later stages of healing in the gingival wounds as compared to skin wounds. The immunostaining level of TGF-β1 protein did not differ significantly in the gingival versus skin wounds.

2.3.6. Gene expression level of β6 integrin and its ligands, TGF-β1 and TGF-β3, in the scarless versus scar-forming wound healing of red Duroc pigs from day 0 (unwounded) to day 49

To evaluate the gene expression pattern of β6 integrin, TGF-β1, and TGF-β3 during scarless gingival and scar-forming skin wound healing of red Duroc pigs we isolated total RNA from the wounds from day 0 (unwounded) to day 49 for real-time PCR. As compared to the unwounded gingiva, β6 integrin expression was significantly upregulated by day 7 post-wounding, remained steady until day 35, and further increased by day 49 (Figure 2.6). In the skin wounds, the expression of β6 integrin increased by about 2-fold in the 7-day-old wounds compared to unwounded tissue, then returned to the level in unwounded tissue by day 14 and remained the same through the later wound time points (Figure 2.6). The expression of TGF-β1

in gingiva did not change until day 49 post-wounding that when approximately 50% increase in TGF-β1 expression compared to unwounded tissue was detected (Figure 2.6).

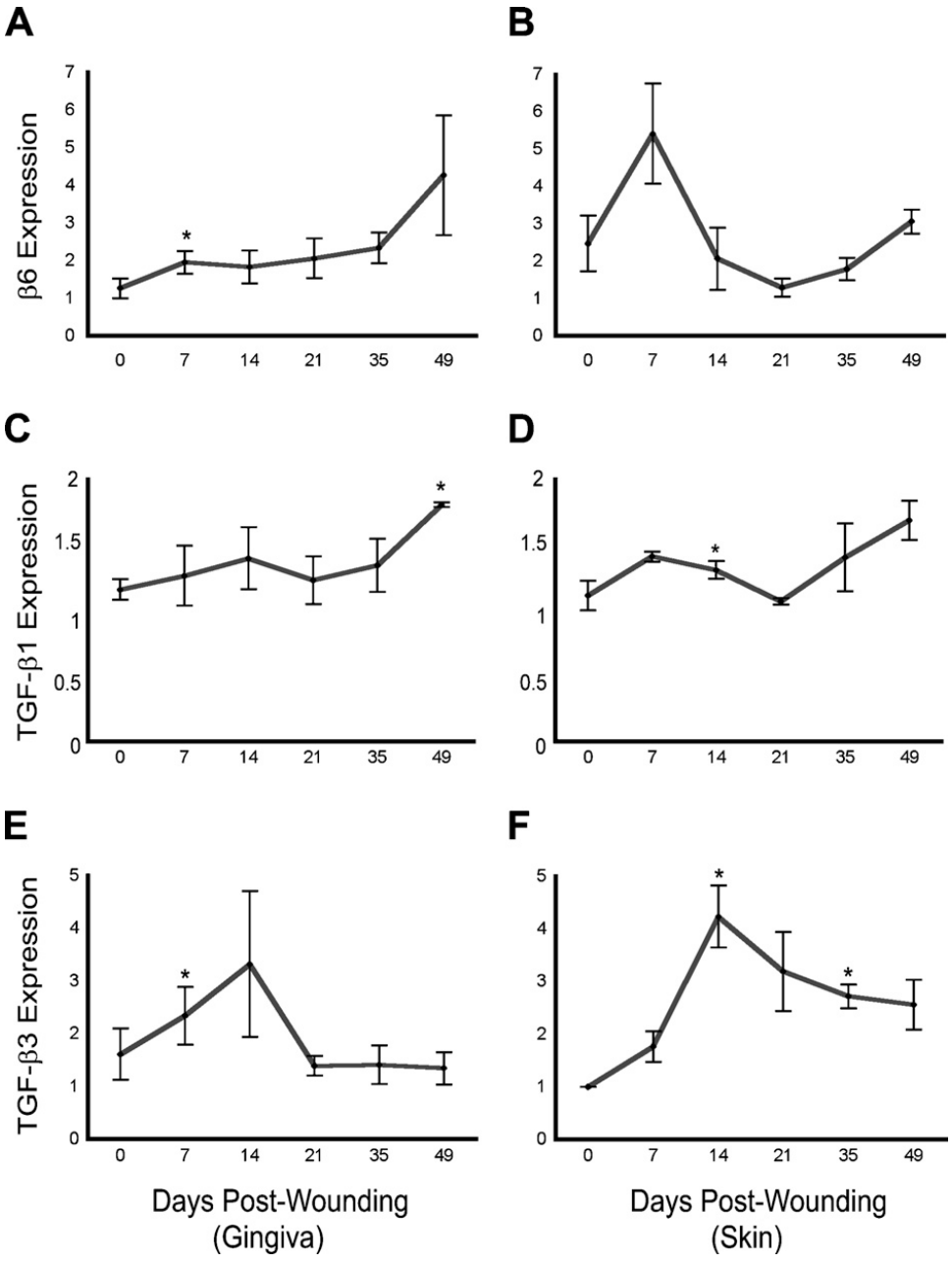

Figure 2.6. Real-time PCR analysis of the expression of mRNA for β6 integrin and its ligands, TGF-β1 and TGF-β3 at different stages of wound healing relative to unwounded tissue in the red Duroc pigs. n=3, p value ≤ 0.05. The error bars represent standard error.

The expression of TGF-β1 in the skin samples showed only moderate changes over the course of healing and followed almost the same pattern as the gingival samples (Figure 2.6). The expression of TGF-β3 was upregulated at day 7 and day 14 in the gingival wounds returning to the level of unwounded tissue at the later time points of healing (Figure 2.6). The expression of TGF-β3 in the skin wounds also increased up to 4-fold by day 14, and then gradually decreased but still remained somewhat higher than unwounded tissue at day 49 (Figure 2.6).

To directly compare the expression level of β6 integrin and its ligands in the gingival and skin wounds, we analyzed the RNA isolated from the gingival and skin samples again in the same real-time PCR experiment. The results showed that unwounded gingiva expressed about twice as much β6 integrin mRNA compared to skin (Figure 2.7). After day 7 post-wounding, the expression of β6 integrin mRNA in the gingival wounds became about 8-fold higher than in the parallel wounds in the skin (Figure 2.7). The expression of TGF-β1 mRNA was about 50% higher in unwounded gingiva versus skin and this difference was also apparent during different time points of wound healing (Figure 2.7). The expression level of TGF-β3 mRNA was about 2-fold in the unwounded gingiva compared to skin (Figure 2.7). This difference decreased over time, and after 21 days of healing, the skin wounds expressed relatively more TGF-β3 mRNA than the gingival specimens (Figure 2.7). Taken together, gingival tissue compared to skin responded to wounding with larger increase in β6 integrin expression and a gradual decline of initially higher TGF-β3 expression while TGF-β1 levels did not significantly change.

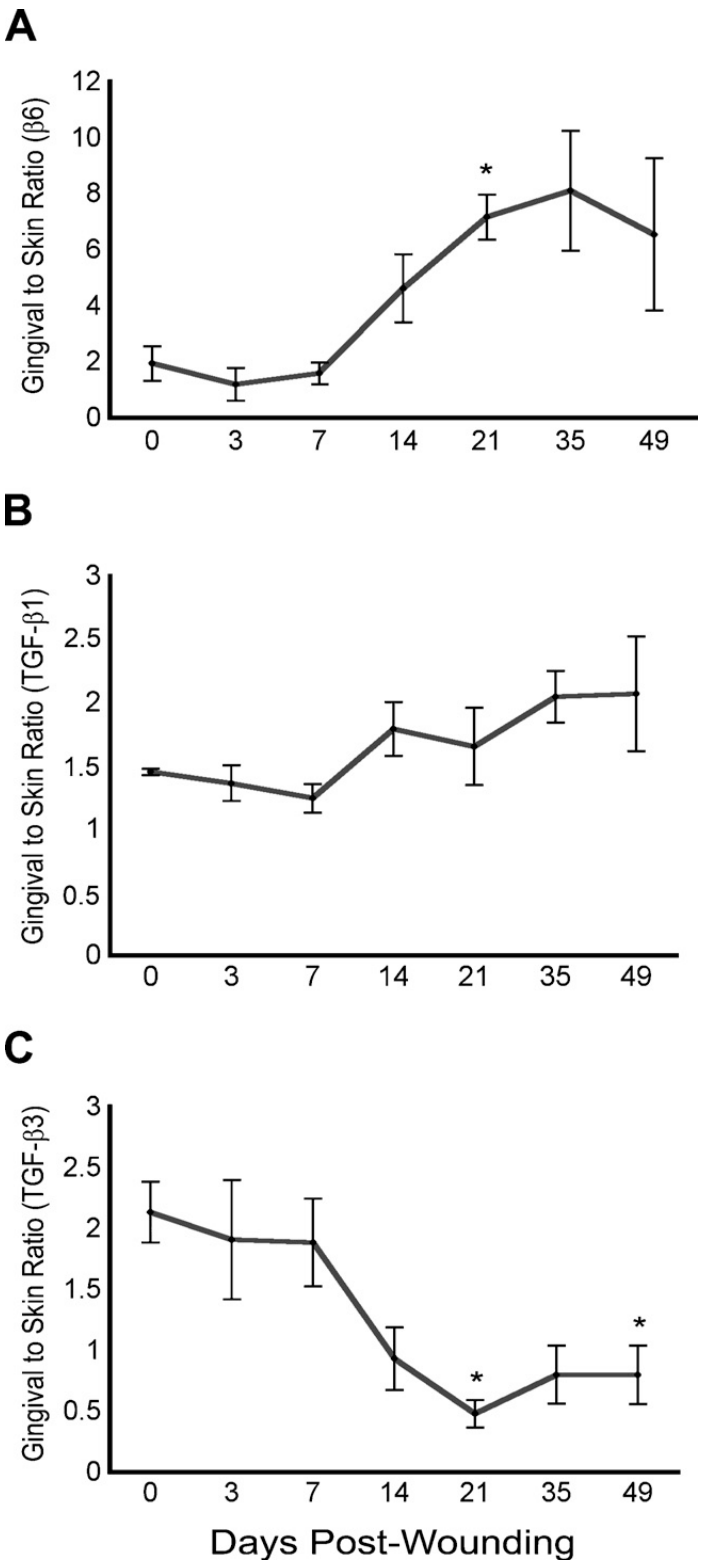

Figure 2.7. Real-time PCR analysis of the expression of β6 integrin, TGF-β1 and TGF-β3 mRNA in unwounded tissue and in the wounds in gingiva relative to skin in the red Duroc pigs. n=3, *p* value < 0.05. The error bars represent standard error.

2.4. Discussion

Oral mucosal wounds heal with minimal scarring unlike scar-forming dermal wounds (Häkkinen et al., 2000; Szpaderska et al., 2003). Integrin αvβ6 is induced during wound healing in both tissues and is an in vitro activator of both fibrogenic TGF-β1 and anti-fibrogenic TGF-β3 (Breuss et al., 1995; Häkkinen et al., 2000b; Keski-Oja et al., 2004). Integrin αvβ6 localizes TGF-β to the cell surface (Munger et al., 1999), suggesting that it can potentially be a local activator of TGF-β isoforms during wound healing. Activation of TGF-β1 by αvβ6 integrin has been shown to play important role in some fibrotic conditions, such as kidney and pulmonary fibrosis (Munger et al., 1999; Kaminski et al., 2000; Ma et al., 2003; Hahm et al., 2007). Furthermore, increased abundance of TGF-β3 relative to TGF-β1 protects healing wounds from scar formation suggesting that there may be a cross-regulation of TGF-β1 and TGF-β3 during wound healing (Shah et al., 1995; Hsu et al, 2001; Ferguson and O'Kane, 2004; Schrementi et al., 2007). In addition to αvβ6 integrin there are other members of αv family that can bind to and activate TGF-β (Wipff and Hinz, 2008). However, most of them are not expressed in epithelial cells (e.g., αvβ3, αvβ8) (Larjava et al., 1996), or have shown to be expressed just temporarily in migratory wound epithelium in early time points of wound healing (e.g., αvβ5) (Clark et al., 1996). In the present study, we showed that αvβ6 integrin could potentially regulate the activity of its ligands, TGF-β1 and TGF-β3, during wound healing as shown by the spatio-temporal colocalization of those molecules at the wound site. Furthermore, integrin αvβ6 and anti-fibrogenic TGF-β3 showed extended accumulation in the scarless gingival wound epithelium as compared with scar-forming skin.

Previous interventional studies have shown that molecular events in very early wound healing may dictate whether tissue scars or not (Ferguson & O'Kane, 2004). For that reason, we first performed transcriptional profiling of molecules involved in TGF-β activation and signaling at the early time points of human gingival wound healing. Our findings showed that the gene expression level of αv integrin along with TGF-β3, LTBP-1 and TGF-β-induced target genes during wound healing (type I and III collagens, fibronectin, CTGF, MMP-1, and MMP-9) were upregulated in 7-day-old human gingival wounds when compared to unwounded tissue. With the immunolocalization studies, the gene micro-array data was confirmed and also detect strong

accumulation of αv and β6 integrins in the wound epithelium was detected. We did not detect expression of β6 integrin by transcriptional profiling most likely because its expression was very low relative to the other transcripts as it was only present in the basal wound keratinocytes at the wound site. Expression of αv integrin was more widespread in the epithelium and accounted most likely also for the expression of αvβ1 integrin in addition to αvβ6 integrin by the wound keratinocytes (Larjava et al., 2002). Interestingly, αvβ6 integrin colocalized with TGF-β1, TGF-β3 and LTBP-1 at or immediately underneath 7-day-old human gingival wound basal epithelium, but not in unwounded tissue. Spatio-temporal colocalization of these molecules suggests a potential for local activation of TGF-βs by αvβ6 integrin during wound healing. Obviously, more biochemical and functional evidence is needed to confirm this possibility. However, based on our findings, CTGF and type I procollagen, molecules that are upregulated by TGF-β signaling, showed both increased immunostaining at the same location where αvβ6 integrin and TGF-βs were present at the basal epithelial cells or immediately underneath. Thus, in this location TGF-β may have been activated by αvβ6 integrin.

We also studied the accumulation and localization of MMP-9 and TSP-1 as other potential activators of TGF-β during wound healing in the 7-day-old human gingival wounds. MMP-9 is both induced by TGF-β and activates it (Salo et al., 1994; Yu and Stamenkovic, 2000). Its up-regulation in gene micro-array analysis and high accumulation at the wound basal membrane zone (BMZ) of the 7-day-old gingival wounds detected by immunostaining suggests a role for MMP-9 in the local activation of TGF-β during wound healing along with other possible activators. Interestingly, high MMP activity has been also linked to scarless fetal wound healing (Dang et al., 2003). TSP-1 did not show, however, apparent changes in the gene array data or in the immunostaining of the 7-day-old wounds when compared to unwounded samples. TSP-1 has been associated with optimal healing in animal models (DiPietro et al., 1996) and collaborates with αvβ6 integrin in TGF-β activation in vivo (Ludlow et al., 2005) but it may be more important for regulation of angiogenesis and granulation tissue formation than in the epithelium (DiPietro et al., 1994).

We have shown previously that during wound healing the expression of αvβ6 integrin is induced in the basal wound epithelium and stays upregulated at least up to day 14 after wounding

(Haapasalmi et al., 1996; Häkkinen et al., 2000b). In the present study, we used another more sensitive antibody against β6 integrin (Huang et al., 1996) that revealed extended expression of β6 integrin in human gingival wounds up to 60 days. High staining intensity of β6 integrin in the fused epithelium of human gingival wounds from day 7 to day 28 was concurrent with maximal accumulation of both TGF-β1 and TGF-β3 in the epithelium. Previous short-term study of rat skin wound healing also showed peak activation of TGF-β1 that occurred before day 7 post-wounding (Yang et al., 1999). Higher accumulation of β6 integrin and TGF-β3 at the later time points in human gingival wound epithelium compared to skin may suggest a preferential activity for TGF-β3 over TGF-β1 in those time points.

We chose red Duroc pigs to compare the expression pattern and localization of β6 integrin, TGF-β1 and TGF-β3 during early and late stages of gingival and skin wound healing. Red Duroc pigs are among tight-skinned animals that share many common wound healing characteristics with humans in terms of scar formation in the skin (Gallant et al., 2004; Gallant-Behm et al., 2005; Zhu et al., 2007). We have demonstrated that similar to humans, gingival wounds in red Duroc pigs heal with minimal scarring (Wong et al., manuscript in preparation). The protein and gene expression pattern of αvβ6 integrin has not been previously compared during gingival and skin wound healing in a long-term study. Interestingly, the localization of αvβ6 integrin in gingival wounds in red Duroc pigs was similar to humans. Integrin αvβ6 was accumulated with high staining intensity in the basal wound epithelium of both gingival and skin wounds from day 7 to day 14. Its accumulation, however, extended to the latest time point studied (49 days post-wounding) in the gingival wounds, but not in the skin. Persistent accumulation of αvβ6 integrin in the gingival as compared with skin wounds was also observed in another study that was performed with larger sized gingival and skin wounds in red Duroc pigs (unpublished data). Significantly higher level of αvβ6 integrin mRNA expression at different time points of gingival wound healing may explain its extended presence in the gingival wounds, but not in those of skin.

The expression and localization of TGF-β isoforms has been mostly studied in the earlier stages of normal wound healing. Many short-term studies in wound healing models (mostly rodents) have shown a peak in TGF-β1 level in the first 24 hours and, in some instances, a

second peak on day 7 post-wounding in skin wounds (O'Kane and Ferguson, 1997). Only a few short-term studies have compared the expression of TGF-β isoforms in oral mucosal versus dermal wound healing (less than three days) using mouse models (Szpaderska et al., 2003; Schrementi et al., 2007). It was found that the steady state expression of TGF-β1 was lower in early stages of gingival wounds when compared to the skin. The level of TGF-β3, however, in the gingiva was three times higher than skin at 24 hours post-wounding and declined afterwards resulting in a higher TGF-β3 to TGF-β1 ratio in earlier stages of gingival wound healing comparing to the skin. Mice are loose-skinned animals with significantly different healing process than humans and the results may not necessarily apply to tight-skinned species, including humans (Hayward et al., 1991). In this study, we investigated the protein and gene expression levels of TGF-β1 and -β3 in unwounded tissues and in the wound time points later than 3 days especially in relation to the expression of β6 integrin. The results showed only small changes in the relative immunostaining intensity and expression of TGF-β1 mRNA at all time points of gingival and skin wounds as compared to unwounded tissue. Interestingly, however, the gene expression of TGF-β1 was higher in all gingival samples when compared to the skin. Expression of TGF-β3 has been shown to peak later in dermal wound healing when TGF-β1 is decreasing (O'Kane and Ferguson, 1997). A recent study showed peak TGF-β1 mRNA expression at 7 days post-wounding in the pig skin wounds while expression of TGF-β3 increased at later time points (Murphy-Ullrich et al., 2007). While a considerable upregulation in TGF-β1 mRNA levels in either gingival or skin wounds was not detected, we did identify that peak mRNA expression of TGF-β3 to be around day 14 in samples of both tissues. The staining intensity of TGF-β3 was generally higher in the normal gingiva and gingival wounds when compared to skin. Late co-localization of TGF-β3 to β6 integrin at the basal epithelium of 49-day-old gingival wounds was also in agreement with the results from human gingival wounds. This suggests that there may be a sustained local activation of the anti-fibrotic TGF-β3 by αvβ6 integrin in the later stages of gingival wound healing resulting in reduced scarring.

Based on knocked out and transgenic animal studies performed in mice, the presence or absence of αvβ6 integrin does not change the normal wound healing process (Häkkinen et al., 2004; AlDahlawi et al., 2006). It is still not clear how αvβ6 integrin functions during normal and abnormal wound healing in tight-skinned species, including humans. It has been shown that in

compromised wound healing conditions, such as in animals under stress and in chronic wounds that develop in β6 integrin over-expressing mice, the activation of TGF-β by αvβ6 integrin may play a role (Häkkinen et al., 2004; AlDahlawi et al., 2006). High levels of αvβ6 expression have also been detected in non-healing chronic wounds in humans (Häkkinen et al., 2004). Considering that TGF-β regulates re-epithelialization, connective tissue regeneration and scar formation during wound healing (Kaminski et al, 2000), and the evidence that epithelial cells have significant role in the development of inflammatory skin lesions (Carroll et al., 1995; Chan et al., 2002), it is possible that the local modulation of TGF-β isoforms by their activators, namely αvβ6 integrin, during wound healing may be important in the wound outcome: regeneration or scar formation. In summary, we found that there was persistent accumulation of αvβ6 integrin in scarless gingival wounds of both humans and red Duroc pigs, but not in the scar-forming skin wounds of the pigs. Peak accumulation of αvβ6 integrin between day 7 and day 28 in human gingival wounds was spatio-temporally concurrent with increased staining intensity of both TGF-β1 and TGF-β3. Late stages of gingival wound healing in the pigs also showed co-accumulation of αvβ6 integrin and TGF-β3 at the basal wound epithelium. TGF-β3 was only detected in 7-day skin wounds of the pigs. TGF-β1 showed steady accumulation in different wound healing time points of both skin and gingiva in red Duroc pigs. Persistent accumulation of αvβ6 integrin in the pig gingival wounds was supported by its long-lasting mRNA expression. Although there was higher local accumulation of TGF-β3 in later stages of wound healing in pig gingival wound epithelium, we found the peak mRNA expression of TGF-β3 to be around day 14 in both gingival and skin wounds. TGF-β1 showed steady expression in both gingival and skin wounds, with generally higher expression level in gingiva comparing to skin. Therefore, based on the findings in this study, we suggest that persistent expression of αvβ6 integrin along with higher local accumulation of TGF-β3 in the later stages of wound healing in the gingival wound basal epithelium may sustain the anti-scarring effects of this TGF-β3 isoform in the gingiva but not in the skin. Appropriate functional studies should be undertaken in the future to show the effectiveness of such local modulation.

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Chapter 3: Integrin αvβ6 Dependent Regulation of Latent TGF-β1 in Keratinocytes[2]

3.1. Introduction

Transforming growth factor β1 (TGF-β1) is a multi-functional cytokine that plays major roles in many physiological and pathological conditions (Nishimura, 2009). In adult tissue, TGF-β1 is critically involved in homeostasis of epithelial cells, endothelial cells, immune cells, and mesenchymal cells (Wipff and Hinz, 2008). Dysregulation of TGF-β1 function results in serious pathological conditions such as fibrosis and cancer (Wipff and Hinz, 2008). Significant amount of TGF-β1 is stored and present in the extracellular matrix (ECM) as latent form (Wipff and Hinz, 2008). Therefore, the spatio-temporal presence of TGF-β1 activators rather than the gene expression level of this cytokine is usually an important determinant of TGF-β regulation and functionality. There are three mammalian homologous isoforms of TGF-β: TGF-β1, -β2, and -β3 (Nishimura, 2009) that share similar receptor complexes (TGF-β receptor II and I) and signaling through either smad-dependent or smad-independent signaling pathways (Derynck and Zhang, 2003; Nishimura, 2009). The well-established smad-dependent signaling pathway starts upon binding of active TGF-β to its receptor complex, followed by phosphorylation of intracellular signaling mediators, smad-2/3. Subsequently, smad-2/3 bind to smad-4 and the entire complex translocates into nucleus and binds to smad-response element, which induces the expression of TGF-β target genes (Nishimura, 2009). Although all mammalian isoforms of TGF-β share similar signaling pathway components, they have individual functions *in vivo* (Nishimura, 2009). Most of TGF-β-mediated pathological conditions are caused by over- or under-activity of TGF-β1 (Nishimura, 2009). Autogenic TGF-β1 is produced by different cell lines, including keratinocytes, which secret TGF-β1 into their environment mostly as large latent complex (LLC) (Barrientos et al., 2008; Koli et al., 2001). The large latent complex of TGF-β1 is composed of the mature TGF-β, the latency associated peptide (LAP), and latent TGF-β binding protein-1 (LTBP-1; Nisimura, 2009). A significant amount of TGF-β1 is stored in the ECM through

[2] A version of this chapter has been submitted for publication: Eslami, A., Koivisto, L., Jiang, G., Owen, G. Rh., Tsao, H., Häkkinen, L., & Larjava, H. Integrin αvβ6 dependent regulation of latent TGF-β1 in Keratinocytes.

LTBP-1 and needs to be released from the latent complex for activation (Nishimura, 2009; Wipff and Hinz, 2008). There are several activators of TGF-β that can dissociate the mature TGF-β from LAP, allowing it to interact with its cell surface signaling receptors (Annes et al., 2003). Integrins have been implicated as major activators of TGF-β *in vivo* (Aluwihare et al., 2009; Yang et al., 2007). Like other αv integrins, αvβ6 integrin binds to RGD motifs in its ligands, including LAP of TGF-β1 and TGF-β3 (Thomas et al., 2006). It has been shown that αvβ6 integrin can activate both TGF-β1 and TGF-β3 through binding to LAP part of LLC (Annes et al., 2002, 2004). Upon binding of αvβ6 integrin to LLC, cytoskeletal traction causes conformational changes, exposing the active molecule of TGF-β to its neighboring receptors (Keski-Oja et al., 2004).

Integrin αvβ6 is an exclusively epithelial cell surface receptor, which has limited expression in adult tissues (Breuss et al., 1993). Its expression, however, is induced during conditions such as wound healing and cancer (Breuss et al., 1995; Häkkinen et al., 2000). The β6 integrin-knockout mouse shares similarities in phenotype with the TGF-β1-knockout animals, including exaggerated lung and skin inflammation, suggesting a role for αvβ6 integrin in the activation of TGF-β1 *in vivo* (Huang et al., 1996). Furthermore, TGF-β1 activation via αvβ6 integrin contributes to renal and radiation-induced fibrosis (Hahm et al., 2007; Puthawala et al., 2008), whereas β6-knockout mice are protected from TGF-β1-mediated lung fibrosis induced by bleomycin (Munger et al., 1999).

During wound healing, keratinocytes produce TGF-β1 and also interact with ECM-bound latent TGF-β1. TGF-β1 promotes keratinocyte migration but inhibits their proliferation (Sivamani et al., 2007, Sheppard, 2006). Accordingly, blocking TGF-β signaling appears to improve full-thickness skin wound re-epithelialization by removing TGF-β-mediated inhibition of cell proliferation (Amendt et al., 2002; Ashcroft et al., 1999). For optimal healing, keratinocytes need, therefore, to be able to manage their microenvironment that contains potentially high levels of ECM-associated TGF-β1 in addition to their own endogenously produced TGF-β1.

In the present study, we investigated the interaction of keratinocytes with either endogenously produced or matrix-stored latent TGF-β1. We found that keratinocyte αvβ6 integrin activates endogenous TGF-β1, resulting in upregulation of TGF-β1-induced target genes, but it also protects the cells from the effects of excess ECM-bound TGF-β1.

3.2. Materials and methods

3.2.1. Cell lines

The human epidermal keratinocyte cell line HaCaT (a generous gift from Dr. Norbert Fusenig, German Cancer Center, Heidelberg, Germany) was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL Life Technologies, Rockville, Maryland, USA) supplemented with 23 mM sodium bicarbonate, 20 mM HEPES, antibiotics (50 mg/ml streptomycin sulfate, 100 U/ml penicillin) and 10% heat-inactivated fetal calf serum (FCS; Gibco BRL). The HaCaT cell line models many of the properties of normal epidermal keratinocytes, is not invasive and can differentiate under appropriate experimental conditions (Boukamp et al., 1988). It should be considered, however, that HaCaT keratinocytes are an immortalized cell line through bearing a mutation in p53 gene and this immortalization may lead to alteration in TGF-β activation or signaling (Lehman et al., 1993).

Chinese hamster ovary cells (CHO K7, ATCC, Manassas, VA, USA) were cultured in the CHO medium containing above-mentioned medium supplemented with 0.2% (w/v) bovine serum albumin (BSA; Saharinen et al., 1996). LTBP-1 producing CHO cells were obtained from Dr. Jorma Keski-Oja, (University of Helsinki, Helsinki, Finland) and routinely grown in the CHO medium supplemented with G418 (350 µg/ml, Gibco-BRL-Geneticin®). In short-term (2-day) experiments, however, no G418 was added. Primary progenitor-like wild-type (WT) and β6 integrin-deficient (β6-/-) mouse keratinocytes were isolated and maintained in complete keratinocyte medium composing of EMEM (Lonza, Walkersville, MD, USA), 9% FCS, 50% fibroblast conditioned medium, 0.05 mM $CaCl_2$, EGF (4 ng/ml) and antibiotics as described previously (Häkkinen et al., 2001). Two separately isolated progenitor-like WT and β6-/- mouse keratinocyte cell lines were used for the experiments in parallel. WT and β6-/- mouse keratinocytes were characterized for their integrin expression, cell spreading, and growth as described below.

3.2.2. Generation and interaction of cells with LTBP-1-rich matrix

3.2.2.1. CHO cell-derived ECM production and characterization

WT or LTBP-1-producing CHO cells (70,000 cells per cm^2) were seeded in cell culture plates (6-well, 12-well or 96-well plates or on glass coverslips placed in 24-well plates as per experimental design) in the CHO medium and allowed to deposit an ECM for 48 hours. Subsequently, the CHO cells were removed by 15 mM EDTA treatment for 30-40 minutes at 37°C as described previously (Annes et al., 2004). The matrices were washed with PBS (phosphate buffered saline) several times and kept in PBS for later use at +4°C no longer than one month.

To measure the total content of TGF-β1 in the matrices deposited by wild type or LTBP-1-producing CHO cells, the matrices were extracted from 12-well plates with cold lysis buffer (50 µl/well) composed of 100 mM n-octyl-β-D-glucopyranoside and 1 µg/ml aprotinin in PBS. The matrices were then collected using a cell scraper (08-773-2, Fisherbrand, Whaltham, MA, USA), and transferred into separate microtubes. Subsequently, the level of TGF-β1 was quantified in each sample using Quantikine human TGF-β1 ELISA kit (DB100-B, R&D Systems, Minneapolis, MN, USA) as per manufacturer's instructions. Using similar method, the level of latent TGF-β1 was assessed in LTBP-1-rich matrices, which were incubated at 37°C with the HaCaT keratinocyte conditioned medium (medium collected from confluent HaCaT keratinocytes grown in serum-free medium for 48 hours) in the absence of cells for 0, 6, 12, and 24 hours.

3.2.2.2. CHO cell-derived extracellular matrices populated with epithelial cells

To assess the level of TGF-β in the CHO cell-derived matrices in the presence or absence of epithelial cells, αvβ6 integrin-expressing HaCaT keratinocytes or wild-type CHO cells (β6 integrin negative cells) were seeded in two different cell densities in 12-well plates (30,000 and 70,000 cells per cm^2) on the LTBP-1-rich or wild-type matrices in the CHO medium overnight. Subsequently, the cells were serum-starved and cultured for another 24 hours. Control matrices were incubated with the conditioned medium from the cells cultured on plastic. Subsequently,

the cells were removed by 15 mM EDTA treatment, the matrices were collected and the level of total TGF-β1 was measured as described above.

In order to test the role of β6 integrin in the interactions of keratinocytes with the CHO cell-derived matrix, HaCaT keratinocytes were seeded on LTBP-1-rich matrices in 12-well plates (70,000 cells per cm^2). The cells were allowed to attach for 2 hours in their regular medium before they were treated with a monoclonal β6 integrin function-blocking antibody (10 μg/ml, 6.3G9, Biogen), a non-functional antibody against β6 integrin (10 μg/ml, 7.8B3, Biogen), or left untreated, and kept in culture for overnight. Subsequently, the medium was replaced with serum-free medium and the antibody treatments were repeated for another 24 hours. At the end of the experiment, the cells were removed by lysis buffer treatment (50 μl per well) for 10 minutes on ice followed by a gentle wash with PBS. Another 50 μl of lysis buffer was added on the matrices that were then released by using a cell scraper and analyzed for TGF-β1 activity by ELISA. Identical experiments were performed using the progenitor-like skin keratinocyte lines isolated from WT and β6-/- mice except that no antibodies were added to the cultures and the cells were kept in culture for up to 48 hours after serum starvation.

3.2.3. Expression of TGF-β1 target genes in keratinocytes

HaCaT cells (70,000 cells per cm^2 in 6-well plates) were cultured on LTBP-1-rich matrices or on monomeric type I collagen (see below) in serum-free DMEM for 2 hours at 37°C to allow cell adhesion. Subsequently, the cells were treated with a β6 function-blocking antibody (10 μg/ml, 6.3G9; Biogen Idec Inc., Cambridge, Massachusetts, USA), a non-function blocking β6 antibody (10 μg/ml, 7.8B3; Biogen Idec), an anti-TGF-β antibody (10 μg/ml, R&D systems) or LAP (1 μg/ml, R&D Systems) to block TGF-β function, or with irrelevant mouse IgG (10 μg/ml, MsIgG, Sigma) as a control, for 24 hours. Three independent experiments were performed except that the experiment with anti-TGF-β antibody for measuring CTGF gene expression was repeated twice. Pooled cell pellets from duplicate wells were collected, and total RNA was extracted using Nucleospin RNA II kit (Macherey-Nagel, Inc., Bethlehem, PA, USA). The synthesis of oligo(dT)-primed cDNA was performed by reverse transcription of 1 μg total RNA using iScript™ Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's standard protocol. Subsequently, amplification of β6 integrin, CTGF, TGF-β1,

TGF-β3, SARA, smad2, and β-actin (as a reference gene) cDNA was performed using SYBR green on MiniOpticon Real-Time PCR Detection System (Bio-Rad). Primer sequences used for cDNA amplification are listed in Table 1. The gene expression levels of all these molecules were normalized to β-actin using the comparative C_T method. Melting curve analyses were performed for all amplifications to verify that only single products were generated from the reactions.

Table 3.1. Primer sequences used for cDNA amplification in gene expression studies in HaCaT keratinocytes.

Primer	Sequence	bp	Accession Number
β6	AATTGCCAACCCTTGCAGTAG	64	NM_000888
	AATGTGCTTGAATCCAAATGTAG		
CTGF	ATGATGTTCATCAAGACCTGTGCCTG	80	NM_001901
	CTTCCTGTAGTACAGGGATTCAAAGATGTC		
TGF-β1	CCCACAACGAAATCTATGACAAG	81	NM_000660
	GCTTCTCGGAGCTCTGATG		
TGF-β3	ACACCAATTACTGCTTCCGCAA	81	NM_003239
	GCCTAGATCCTGTCGGAAGTC		
SARA	GGATACCTTGCCAAGTCCA	78	AF104304
	CCTCAAGGAATCCATGTTCTC		
Smad2	ACAGGCTCTCCAGCAGAA	82	NM_005901
	TCTGAGTAAGTAACTGGCTGTAA		
β-actin	CTGTGGCATCCACGAAAC	88	NM_007393
	CAGACAGCACTGTGTTGG		

3.2.4. Western blotting

HaCaT keratinocytes were seeded (70,000 cells per cm^2 in 6-well plates) on wild-type CHO cell-derived matrices, LTBP-1-rich matrices or on type I collagen in serum-free medium for 0, 1, 2 or 24 hours. At the designated time points, cells were lysed in the SDS sample buffer and collected into microtubes for Western blotting. Moreover, cells seeded on LTBP-1-rich matrix in the presence or absence of the β6 integrin function-blocking antibody (10 µg/ml, 6.3G9) or irrelevant mouse IgG (10 µg/ml) for 1 hour were lysed and collected for Western blotting. Cell lysates were separated by SDS/PAGE, transferred onto Hybond ECL membrane (Amersham, Little Chalfont, Buckinghamshire, UK) and immunoblotted with primary antibodies against phospho-smad2 and total smad2 (Cell Signaling Technology, Beverly, MA, USA). Peroxidase-conjugated IgGs (Santa Cruz Biotechnology) were used as secondary antibodies. Detection was performed with Amersham's ECL Advance Western Blotting Detection kit. Relative band intensities were measured from images captured by digital camera using NIH Image software (http://rsb.info.nih.gov/nih-image/).

3.2.5. Immunofluorescence staining

The distribution and co-accumulation of β6 integrin and LAP, a part of latent TGF-β complex and the binding partner of αvβ6 integrin, was studied in HaCaT keratinocytes treated with Fc-labeled latency-associated peptide (LAP-Fc) over time. To this end, HaCaT keratinocytes were seeded on 12 mm glass coverslips (20000 cells per cm^2 in 24-well plates) and incubated overnight in their regular medium. Then, the medium was replaced with serum-free medium and the cells were cultured for another 24 hours. Subsequently, the cells were cooled down to 4^oC on ice for 15 minutes to limit endocytosis. Nonspecific binding sites were blocked for 15 min in blocking solution [10% BSA/0.1% Glycine (w/v) in PBS] at 4^oC. The cells were then washed gently three times with cold PBS, and subsequently, treated with pre-cooled (4^oC) 2.5 µg/ml LAP-Fc (Biogen Idec) on ice for 15 minutes. This point was considered the time point 0. The cells were then incubated at 37^oC for 10, 30, and 60 minutes, before removing LAP-Fc from the cultures by placing the samples on ice and rinsing them six times with cold PBS. The samples were then fixed with 4% (v/v) formaldehyde in PBS containing 5% (w/v) sucrose.

For immunofluorescence staining, the fixed cells were permeabilized with 0.5% (v/v) Triton X-100 in PBS for 5 minutes followed by three washes with PBS. The samples were blocked with 1% (w/v) BSA/0.1% (w/v) glycine and 0.2% (v/v) Triton X-100 in PBS for 30 minutes at room temperature and then incubated with Alexa goat anti-human 488 IgG (H+L) (1:100 dilution, Molecular Probes, Inc., Eugene, OR, USA) for one hour at room temperature, followed by three rinses with washing solution containing 1% (w/v) BSA/0.1% (w/v) glycine and 0.2% (v/v) Triton X-100 in PBS. Subsequently, the cells were incubated with a primary antibody against β6 integrin (1:100, monoclonal, 6.2 E5 murine Fc, clone 13-1, Biogen) for overnight at 4°C. After washings the cells were incubated with Alexa anti-mouse 594, $F(ab')_2$ fragment of goat anti-mouse (H + L) (1:100 dilution, Molecular Probes) for one hour at room temperature. Next, the samples were washed twice with the washing solution once with the washing solution containing 40 µg/ml of mouse normal serum (to remove any unbound secondary antibody) and once with PBS and mounted in Prolong antifade (Invitrogen, Grand Island, NY). All samples were imaged under oil immersion with the magnification of x60 or x100 of a Zeiss Axio Obsever Z.1 (Carl Zeiss, Jena, Germany). The Alexa 488 labeled samples were imaged using the EGFP filter and Alexa 594 using the Rhodamine filter. Images were captured with an AxioCam digital camera (Zeiss) using Axiovision software (Zeiss) and saved as 12-bit grayscale images in TIF file format. Each image was then processed for merging, which involved digitally applying a color map for each filter (green for EGFP and red for Rhodamine) onto the grayscale image. Both colored images were then merged using image-matching software available on Axiovision.

3.2.6. Measurement of integrin expression

Mouse keratinocytes were analyzed for their cell surface expression of α5, α6, and β1 integrin subunits as well as the αvβ6 integrin complex, as described previously (Riikonen et al., 1995). Briefly, the cells were seeded in 60-mm cell culture plates (1,000,000 cells per plate) and cultured for two days, trypsinized, and immunostained with antibodies against mouse α5 (MAB1984, Chemicon, Temencula, CA, USA), β1 (10 µg/ml, MAB1997, Chemicon), α6 (MA6, a kind gift from Dr. Bosco Chan, Robarts Research Institute, London, ON, Canada), and αvβ6 integrins (20 µg/ml, MAB2077Z, Chemicon). Species-specific Alexa Fluor™ 488 IgGs (Molecular Probes) were used as secondary antibodies. The immunolabeled cells were analyzed

using FACSCalibur flow cytometer (Becton Dickinson, Oxhard, CA, USA). Control immunostainings were performed using secondary antibodies only.

Expression of αv integrin was assessed in wild-type and β6-/- mouse keratinocytes by Western blotting. The cells were grown to 70% confluency and then lysed in tris-buffered saline (TBS, pH 7.6) containing proteinase inhibitors (Complete protease inhibitor cocktail, Roche Diagnostics, Indianapolis, IN, USA) and 100 mM n-octyl-D-glucopyranoside. Western blotting was performed using a primary antibody against αv integrin (sc-6618, Santa Cruz). Antibodies against β6 integrin subunit (sc-6632, Santa Cruz) and β-actin (Ab8227, Abcam, Cambridge, MA, USA) were used as controls. Species-specific peroxidase-conjugated IgGs (Santa Cruz Biotechnology) were used as secondary antibodies. Detection was performed with Amersham's ECL kit.

3.2.7. Cell spreading assay

Cell culture wells were coated with either 20 µg/ml of bovine fibronectin (from bovine plasma, Chemicon) or 1 µg/ml of monomeric type I collagen (Vitrogen 100, 2.9 mg/ml of 0.012 N HCl, Cohesion, Palo Alto, CA, USA) in PBS^{+} (PBS containing Ca^{2+} and Mg^{2+}, pH 7.4) as described previously (Koivisto et al., 1999). Wild-type and β6-/- mouse keratinocytes were seeded in 4-well culture plates in serum-free EMEM supplemented with 0.05 mM $CaCl_2$. Cells were allowed to attach and spread for up to 60 minutes at 37°C. Cell spreading was quantified as described previously with some modifications (Koivisto et al., 1999).

3.2.8. Cell proliferation assay

Wild-type or β6-/- mouse keratinocytes (2000 cells per well in 96-well cell culture plates) were seeded in triplicates in non-coated or LTBP-1-rich matrix-coated wells. The cells were cultured for up to eight days. Cell proliferation was determined by Promega cell proliferation kit as per manufacturer's instructions (CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI, USA).

3.2.9. Gene expression profiling of wild-type and β6-/- mouse keratinocytes on LTBP-1-rich matrix

Two parallel cell isolates of progenitor-like WT or β6-/- mouse keratinocytes were seeded on either LTBP-1-rich matrices or monomeric type I collagen-coated plates in triplicate to the density of 70,000 cells per cm^2. The cells were cultured in complete keratinocyte medium for 24 h and then switched to a serum-free medium. After 24 hours, the cells were collected and total RNA extracted as above (section ***2.3***). Gene expression profiling was performed at the Prostate Center Microarray Facility (Vancouver, BC, Canada) using Agilent whole mouse genome micro-array according to Agilent protocols (Agilent Technologies, Santa Clara, CA, USA). Scanning and analysis was performed on the Agilent DNA micro-array scanner with Feature Extraction Software (Agilent Technologies). Statistical and clustering analyses were performed using GeneSpring (Agilent Technologies) data mining tool. From the original data set, the genes whose signal intensities varied significantly among the two different cell isolates were excluded based on the *p* value ≥0.05. From the revised gene list, fold-change filtering (≤ 0.5 or ≥ 2, at the *p* value ≤ 0.05) was used to identify probe sets whose signal intensity varied significantly among different groups. Then, the average of the data from each set of two biological replicates (two replicates of WT and β6-/- keratinocytes) on each substrate was calculated. Moreover, the revised gene profiles of the experimental groups were analyzed and visualized by hierarchical clustering. Finally, virtual pathway analysis was performed by the Ingenuity Pathway Analysis database (Ingenuity Systems).

3.2.10. Statistical analysis

Student *t*-tests were performed to verify whether the content of TGF-β1 was significantly different in LTBP-1-rich versus WT CHO matrices. Moreover, student *t*-tests were applied to determine significant differences between wild-type and β6-/- mouse keratinocyts in terms of their spreading, growth rate, content of TGF-β1 in LTBP-1-rich matrix populated with either cell line, and gene expression profiling. Microsoft Excel software was used for performing *t*-test.

One-way analysis of variance (ANOVA) and Dunnett posttest were performed to analyze the data generated by PCR in terms of any significant differences between the treatment groups when compared to the control untreated group. For all other multiple comparisons one-way

ANOVA and Tukey's posttest were performed to determine any statistically significant differences. ANOVA and posttests were performed using SPSS-10. In all comparisons a *p* value ≤ 0.05 was considered as statistically significant.

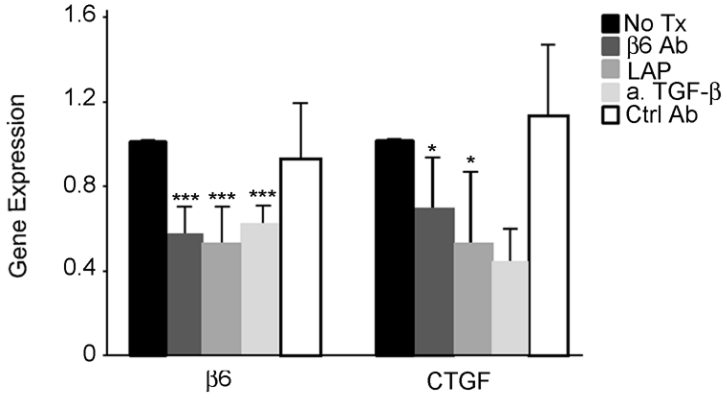
3.3. Results

3.3.1. Interaction of HaCaT keratincocytes with endogenous TGF-β1 is regulated by αvβ6 integrin

Autogenic TGF-β1 is produced by many cell lines, including keratinocytes (Barrientos et al., 2008), which secrete TGF-β1 into their micro-environment mostly as a large latent complex (LLC; Koli et al., 2001). Through studying the gene expression level of key targets of TGF-β1 pathway, we investigated the role of αvβ6 integrin in regulation of TGF-β activity in HaCaT keratinocytes. For the target genes, we selected β6 integrin, connective tissue growth factor (CTGF), TGF-β1, TGF-β3, smad2 and SARA (the smad anchor for receptor activation) that are downstream targets of TGF-β signaling or involved in regulation of TGF-β activity. Expression of β6 integrin was significantly (about 40-50%) down-regulated in HaCaT keratinocytes in which TGF-β activation was suppressed or blocked by a β6 integrin function-blocking antibody, LAP or a TGF-β neutralizing antibody (Figure 3.1.A). The control antibody had no effect.

The expression of CTGF was similarly down-regulated by either blocking the αvβ6 integrin or TGF-β activity by LAP treatment (Figure 3.1.A). CTGF gene expression was also down-regulated by anti-TGF-β antibody treatment although statistical analysis was not possible due to small sample size (n=2 experiments). Expression of TGF-β1, TGF-β3, smad2 and SARA were all significantly stimulated by the β6 integrin antibody treatments (Figure 3.1.B). Although the expression of these molecules was also stimulated by LAP treatment, the increase did not reach statistical significance (Figure 3.1.B). In summary, we showed that αvβ6 integrin plays an important role in the activation of autogenic TGF-β1 signaling pathway in HaCaT keratinocytes. Furthermore, blocking αvβ6 integrin function may lead to compensatory increases in expression of TGF-βs and SARA.

A)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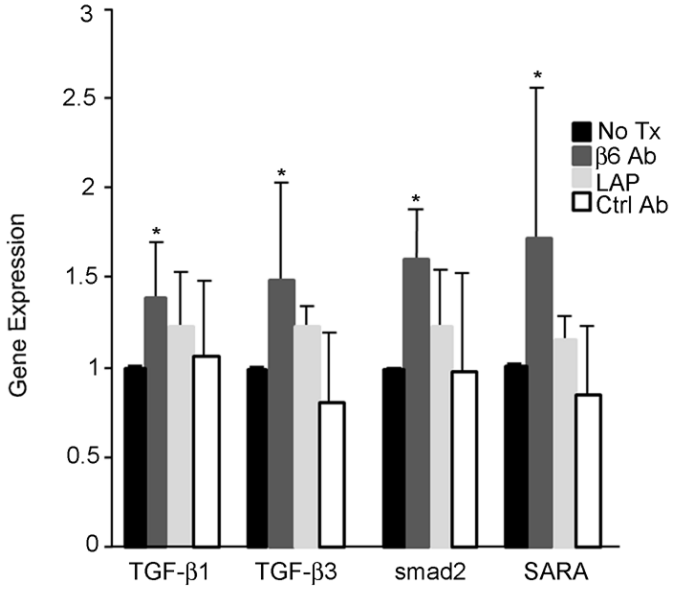
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smad2
SARA

Figure 3.1. Real-time PCR analysis of mRNA for autogenic TGF-β1-induced or -related genes in HaCaT keratinocytes. HaCaT keratinocytes were seeded on type I collagen for 2 h in serum-free medium to allow cell adhesion followed by addition of β6 function-blocking antibody (β6 Ab), control antibody (Ctrl Ab), latency associated peptide (LAP), or anti-TGF-β antibody (a. TGF-β) for 24 h. Total RNA was isolated and the expression level of TGF-β1 target genes, β6 integrin and CTGF (A), as well as other genes involved in TGF-β signaling pathway including TGF-β1, TGF-β3, smad2, and SARA (B) was assessed. All gene expression levels were normalized to β-actin reference gene expression level. Three separate experiments (CTGF expression level in the presence of a. TGF-β, N=2), * $0.01<p$ value<0.05; *** p value<0.001. Error bars represent standard deviations (SDs) except for the bar that shows the a. TGF-β in the CTGF group (lightest grey), in which the error bar shows the range (due to the small sample size).

3.3.2. Keratinocyte αvβ6 integrin is involved in depletion of matrix-bound latent TGF-β1

Having shown that αvβ6 integrin regulates signaling activity of the relatively small amount of endogenous TGF-β1 in keratinocytes, we investigated the role of this integrin in regulating the activity of latent TGF-β1 present much more abundantly in the ECM. For that purpose, we produced LTBP-1-rich matrices deposited by LTBP-1-transfected CHO cells. To characterize these matrices, we measured the level of latent TGF-β1 incorporated into matrix by ELISA. Matrices deposited by wild-type CHO cells (WT-CHO) were used as a control. It was found that LTBP-1 producing CHO cells deposited relatively high level of latent TGF-β1 into their matrix (Figure 3.2.A). The level was about 23-fold higher compared to the matrix of the WT cells (Figure 3.2.A). Negligible amount of active TGF-β1 was found in these matrices (not shown). Next, the level of latent TGF-β1 was assessed in original LTBP-1-rich matrices and in the matrices which were incubated at 37°C with the HaCaT keratinocyte conditioned medium in the absence of cells for 6, 12, and 24 hours (Figure 3.2.B). The level of total TGF-β1 did not show any significant changes at different time points, demonstrating that the matrix-bound latent TGF-β1 is not activated and removed by enzymes and potential other activators produced by the keratinocytes into the medium (Figure 3.2.B). We then investigated whether the presence of αvβ6 integrin-positive keratinocytes will influence the level of latent TGF-β1 in the LTBP-1-CHO matrix.

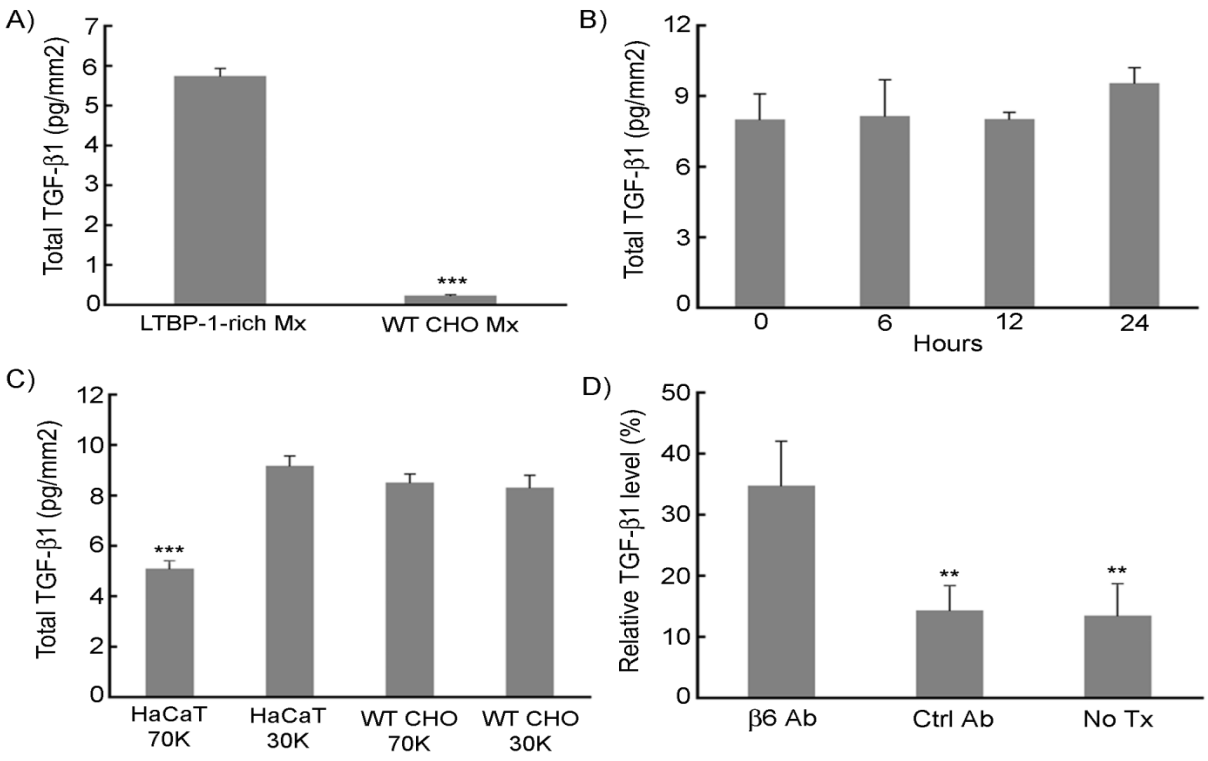


Figure 3.2. Characterization of latent TGF-β1 matrix and αvβ6 integrin-mediated depletion of matrix-bound latent TGF-β1. A) The level of latent TGF-β1 was measured in the matrix produced by culturing LTBP-1-expressing CHO cells (LTBP-1-rich Mx). Matrix deposited by wild-type CHO cells (WT CHO Mx) was used as a control (N=3). B) The level of latent TGF-β1 was assessed in LTBP-1-rich matrices, which were incubated at 37°C with the HaCaT keratinocyte conditioned medium in the absence of cells for the indicated time points up to 24 hours (N=3). C) The level of latent TGF-β1 was measured in the matrix portion of the cultures containing HaCaT keratinocytes (αvβ6 integrin expressing) seeded on LTBP-1-rich matrices in two different cell densities, 30,000 (30K) and 70000 (70K) cells per cm^2, in 12-well plates. The cells were seeded in CHO medium overnight followed by a 24 hours of serum deprivation. The cells were removed by EDTA treatment and the matrix portion was assessed for the level of latent TGF-β1. Wild-type CHO cells (β6 negative) were used as controls (N=3). D) The level of latent TGF-β1 was assessed in the HaCaT keratinocytes (70K cells per cm^2 in 12-well plates) seeded on LTBP-1-rich matrices in the presence and absence of β6 integrin function-blocking or control antibody. The cells were kept in their growth medium for 2 hour to allow cell attachment before antibody treatment for overnight. Subsequently, the medium was replaced with serum-free medium supplemented with the respective antibodies, and the cells were kept in culture for another 24 hours. Subsequently the matrix portion was collected for the assessment of TGF-β1 by ELISA (N=4); ** $0.001<p$ value<0.01; *** p value<0.001. Error bars represent standard deviations.

Keratinocytes were seeded on the matrices in two different cell densities, 30,000 (30K) and 70,000 (70K) cells per cm^2 in 12-well plates in the CHO medium for overnight. Subsequently, the cells were serum-starved and cultured for another 24 hours (Figure 3.2.C). In the presence of HaCaT keratinocytes in high density (70,000 cells per cm^2), TGF-β1 was significantly depleted from the LTBP-1-rich matrices while the lower density had little effect on the matrix bound TGF-β1 (Figure 3.2.C). To investigate whether this depletion of TGF-β1 was keratinocyte-dependent, we repeated the experiment in the presence of αvβ6 integrin-negative WT CHO cells that produce only very small amounts of autogenous TGF-β in the same cell densities. These cells did not affect the level of latent TGF-β1 in the matrix regardless of their density (Figure 3.2.C). To test whether the depletion of TGF-β1 from LTBP-1-rich matrices was αvβ6 integrin-dependent, we treated HaCaT keratinocytes seeded in high density (70,000 cells per cm^2) on LTBP-1-rich matrices with the β6 integrin function-blocking antibody or a control antibody. The β6 integrin function-blocking antibody potently blocked the decay of latent TGF-β1 from LTBP-1-rich matrices populated with HaCaT keratinocytes (Figure 3.2.D). The control antibody had no blocking effect (Figure 3.2.D) In summary, we showed that keratinocytes deplete the latent TGF-β1 from the LTBP-1-rich matrices by cell density- and αvβ6 integrin-dependent mechanisms.

3.3.3. Rgulation of matrix-bound TGF-β1-induced gene expression in keratinocytes

As described above, we found that latent TGF-β1 was significantly decayed from the matrix by HaCaT keratinocytes seeded on LTBP-1-rich matrices. To test whether this decay was associated with altered TGF-β1 signaling, we studied the expression level of the genes involved in the TGF-β1 signaling pathway in the presence or absence of a β6 integrin function-blocking antibody or LAP that blocks TGF-β function. Contrary to the results with keratinocytes in the presence of endogenous latent TGF-β1 (Figure 3.1), no significant differences were found between the treatment and control groups (Figure 3.3.A), suggesting that αvβ6 integrin did not affect the expression of the TGF-β1-induced or -related genes in HaCaT keratinocytes seeded on LTBP-1-rich matrices. We then tested whether the smad2 (TGF-β1 signaling mediator) is

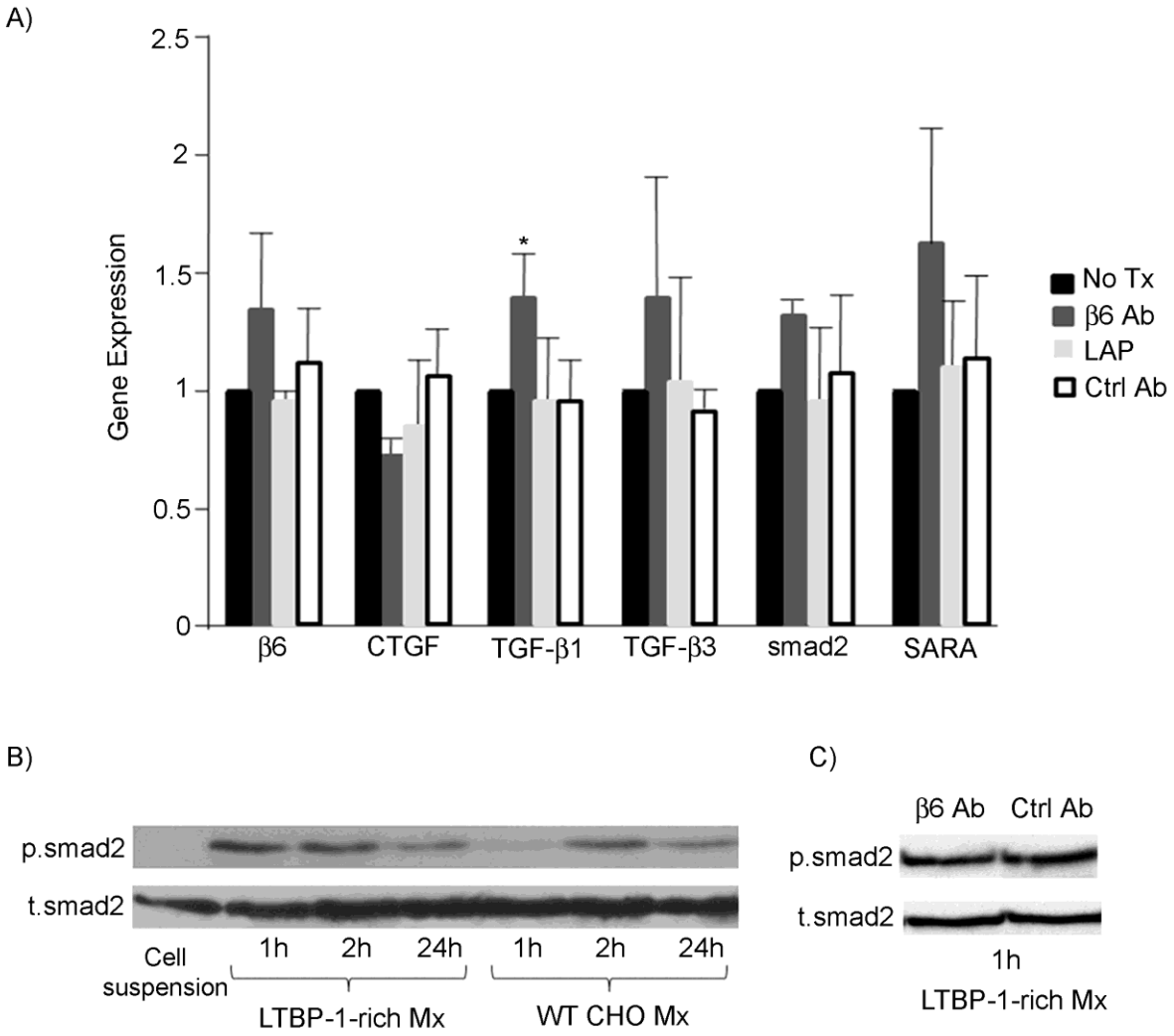

A)
2.5
2
1.5
1
0.5
0
Gene Expression
*
β6
CTGF
TGF-β1
TGF-β3
smad2
SARA
No Tx
β6 Ab
LAP
Ctrl Ab
B)
p.smad2
t.smad2
Cell suspension
1h
2h
24h
1h
2h
24h
LTBP-1-rich Mx
WT CHO Mx
C)
β6 Ab
Ctrl Ab
p.smad2
t.smad2
1h
LTBP-1-rich Mx

Figure 3.3. Regulation of TGF-β1 signaling by HaCaT keratinocytes on latent TGF-β1 matrix. A) The expression level of the genes involved in the TGF-β1 signaling pathway in HaCaT keratinocytes. HaCaT cells were seeded on the matrix deposited by LTBP-1-producing CHO cells for 2 h followed by a 24 h treatment with the β6 function-blocking antibody (β6 Ab), control antibody (Ctrl Ab), or LAP in serum free conditions. Total RNA was isolated and gene expression relative to β-actin reference gene was assessed using real-time PCR. N=3 separate experiments. Error bars represent standard deviations. B) Smad phosphorylation in HaCaT keratinocytes seeded on LTBP-1-rich or WT CHO matrices. HaCaT keratinocytes were seeded on LTBP-1-rich or WT CHO matrices in serum-free condition for 0 (cell suspension), 1, 2, and 24 hours. In each indicated time point, the cells were lysed, and the level of total smad2 (t.smad2) and phosphorylated smad2 (p.smad2) was measured by Western blotting. A representative blot is presented. C) Smad phosphorylation in HaCaT keratinocytes seeded on LTBP-1-rich matrix in the presence or absence of β6 integrin function-blocking antibody. HaCaT keratinocytes were allowed to attach to LTBP-1-rich for 10 minutes at room temperature in no-serum condition before adding β6 function-blocking antibody or irrelevant mouse IgG. Following the antibody treatment, cells were incubated at 37°C for additional 50 minutes. The level of smad2 phosphorylation was evaluated in collected cell lysates by Western blotting. The experiment was repeated four times. A representative blot is presented.

activated in the presence of these matrices and whether the activation is αvβ6 integrin dependent. Smad2 was activated in keratinocytes seeded both on the wild-type and LTBP-1-rich CHO cell matrices while no activation was observed when cells were seeded on monomeric type I collagen (data not shown) or in cells in suspension (Figure 3.3.B). Significantly more activation was observed at 30 min and 1 h time points in cells seeded on the latent TGF-β1 matrix compared to the matrix produced by the wild-type cells (Figure 3.3.B). However, the β6 integrin blocking antibody had no effect on the smad2 activation in these conditions (Figure 3.3.C).

3.3.4. LAP regulates αvβ6 integrin localization in keratinocytes

It has been shown that β6 integrin binds to and is internalized together with LAP, which is the binding partner of β6 integrin and a part of latent complex of TGF-β1 (Weinreb et al., 2004). To study the interaction and possible internalization of β6 integrin together with latent complex of TGF-β1 in keratinocytes, we treated HaCaT keratinocytes with LAP conjugated to Fc portion of human IgG (LAP-Fc). The cells were permeabilized with triton X-100 after fixation to allow efficient intracellular entry of antibodies. At +4^{o}C (0 minutes), β6 integrin showed diffuse plasma membrane localization with occasional presence at focal adhesions (Figurer 3.4). When the cells were brought to 37^{o}C, β6 integrin started to accumulate in focal adhesions that were visible as early as 10 minutes after LAP treatment (data not shown). At 30 to 60 minutes post LAP treatment at 37^{o}C, β6 integrin was accumulated in focal adhesions and was also present in intracellular vesicles (Figure 3.4). The LAP co-localized with β6 integrin after 30 minutes of incubation with LAP at 37^{o}C in focal adhesions and inside the cells (Figure 3.4). This co-localization was more prominent inside the cells than in focal adhesions 60 minutes after LAP treatment. Parallel cultures of HaCaT keratinocytes not treated with LAP-Fc served as control and were stained for β6 integrin after above-mentioned time points. No significant change was observed in the distribution of β6 integrin from time 0 to 30 minutes time point and β6 integrin was only infrequently localized to the focal adhesion structures at 60 minutes (Figure 3.4). LAP-Fc was only detected between the cells over time when the cell membrane was not permeabilized by triton X-100 treatment after cell fixation (unpublished data). This finding supports the intracellular localization of LAP-Fc. The β6 integrin localization on the cell periphery formed similar shapes as for the vinculin staining pattern in focal adhesions (unpublished). In summary,

αvβ6 integrin was dynamically redistributed in HaCaT keratinocytes and co-accumulated with LAP both in focal adhesions and intracellular vesicles.

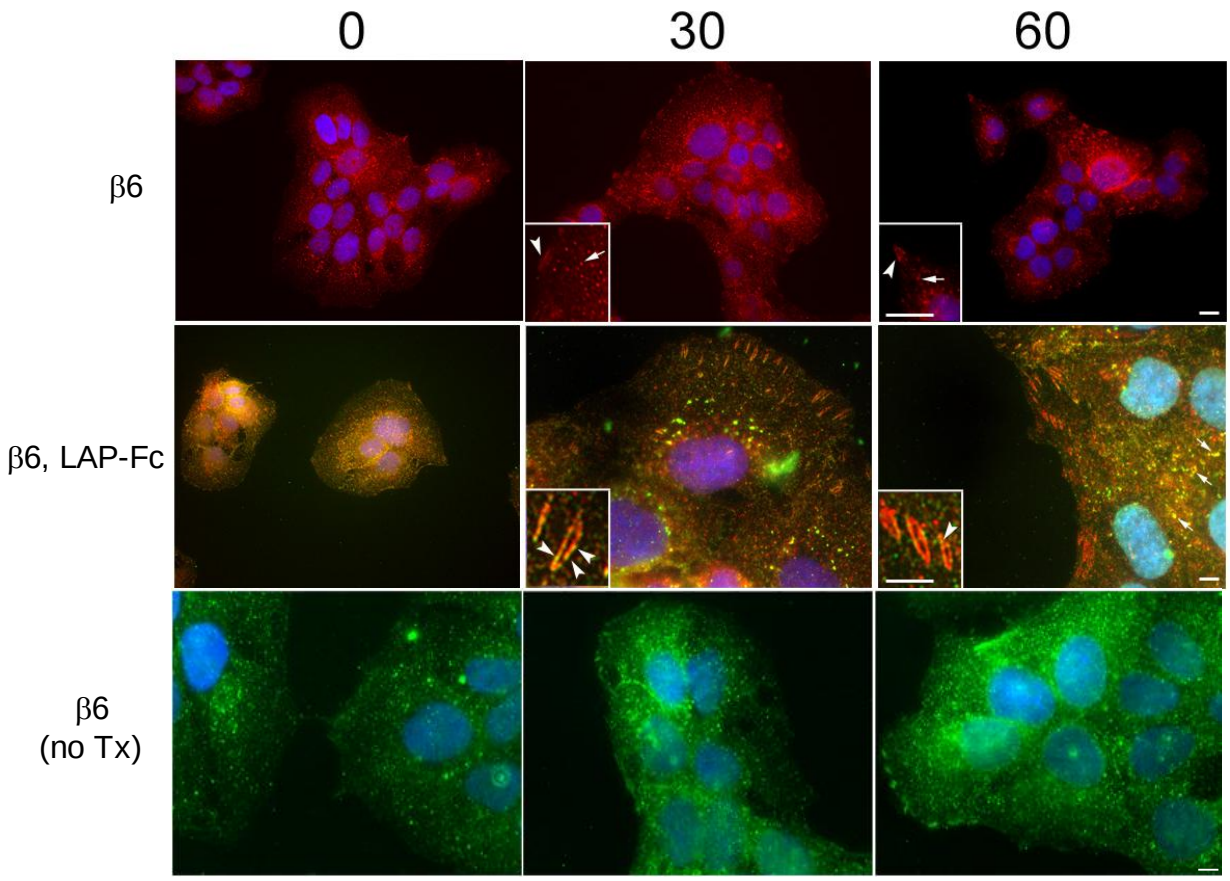


Figure 3.4. Localization of αvβ6 integrin and LAP in HaCaT keratinocytes treated with LAP conjugated to an Fc portion of human IgG. HaCaT keratinocytes were seeded for 24h in DMEM containing 10% FBS and then for a further 24h in serum-free DMEM. Prior to incubating the cells with LAP-Fc they were cooled to 4^{o}C for 15 minutes to inhibit endocytosis. Following blocking with 0.1% BSA in PBS for 15 minutes and rinsing in PBS at 4^{o}C, LAP-Fc was added to the cells and kept for 15 minutes at 4^{o}C (this point was regarded as 0 minute) and then for 30 and 60 minutes respectively at 37^{o}C. The incubation periods were stopped by cooling on ice then rinsing in cold PBS, followed by formaldehyde fixation. Upper panel (X60): Fixed cells were treated with appropriate primary antibody against β6 integrin followed by incubation with fluorescent-conjugated secondary antibody, as shown in red color. In the magnified pictures, arrowheads represent localization of β6 integrin at focal adhesions. Arrows point to the intracellular localization of β6 integrin. Magnification bar=10 μm. Middle panel (X100): LAP and β6 integrin were localized by incubating with secondary antibodies conjugated to fluorescent probes- green for LAP and red for β6 integrin. The colocalization of LAP and β6 integrin (orange) is apparent in the middle panel at focal adhesions (shown by arrow-heads in the magnified pictures) at the 30-minute time point, and to a lesser degree, at the 60-minute time point. Arrows represent intracellular co-localization of β6 integrin and LAP at 60 minutes time point. Lower panel (X100): Parallel cultures of HaCaT keratinocytes not treated with LAP-Fc served as control and were stained for β6 integrin in green color. Magnification bar=3 μm.

3.3.5. Characterization of progenitor-like β6 integrin deficient mouse keratinocytes

To further investigate the αvβ6 integrin-mediated decay of latent TGF-β1 from the matrix, we isolated two independent cell lines of progenitor-like β6 integrin deficient (β6-/-) and WT mouse keratinocytes, as previously described (Häkkinen et al., 2001). To characterize the cell lines, we studied their integrin profiles, cell growth and spreading. As expected, β6-/- mouse keratinocytes did not express αvβ6 integrin, while relatively strong expression of αvβ6 integrin was detected in WT mouse keratinocytes by flow cytometry (Figure 3.5.A). These results were confirmed by Western blotting (Figure 3.5.B). The β6-/- keratinocytes also exhibited reduced level of αv integrin protein (Figure 3.5.B). To rule out any compensatory changes in other integrins, we studied the cell surface expression of α5, α6, and β1 integrins by flow cytometry. The cell surface expression of these integrins was not significantly altered in the β6-/- keratinocytes as compared to WT cells. The antibodies that we applied to detect some other integrin subunits with possible roles in the regulation of TGF-β in keratinocytes (*e.g.*, α3, β5, and β8) did not lead to specific results. However, reduced levels of αv integrin in β6-/- mouse keratinocytes in comparison to the WT cells may suggest that the elimination of the β6 subunit was not compensated by up-regulation of other αv integrins – including αvβ5 and αvβ8 – in these cells. Although the level of β1 subunit was the same in both cell lines, the possible alterations in cell surface expression of different binding partners of β1 integrin in activated keratinocytes should also be considered. The α3β1 integrin is one of such integrin combinations that could influence TGF-β activity and signaling in different ways. It may enforce TGF-β activity through upregulation of MMP-9 as shown by Lamar et al. (2008) or enhance TGF-β signaling by downregulating smad7 in keratinocytes (Reynolds et al., 2008). To assess function of cell surface integrins, we studied cell spreading on fibronectin (ligand for αv and β1 integrins) and collagen (ligand for β1 integrins). β6-/- keratinocytes showed reduced spreading on fibronectin but not on type I collagen (not shown) when compared to WT cells (Figure 3.5.C). Next, proliferation of β6-/- and WT keratinocytes seeded on culture dish plastic were compared during exponential growth. β6-/- cells showed significantly lower cell numbers at day one but they reached the level of WT cells at the later time points (Figure 3.5.D). Taken together β6-/-

mouse keratinocytes lacked αvβ6 integrin expression but had no major changes in their other integrins, showed deficiency on cell spreading on fibronectin and mildly reduced initial cell growth compared to WT cells.

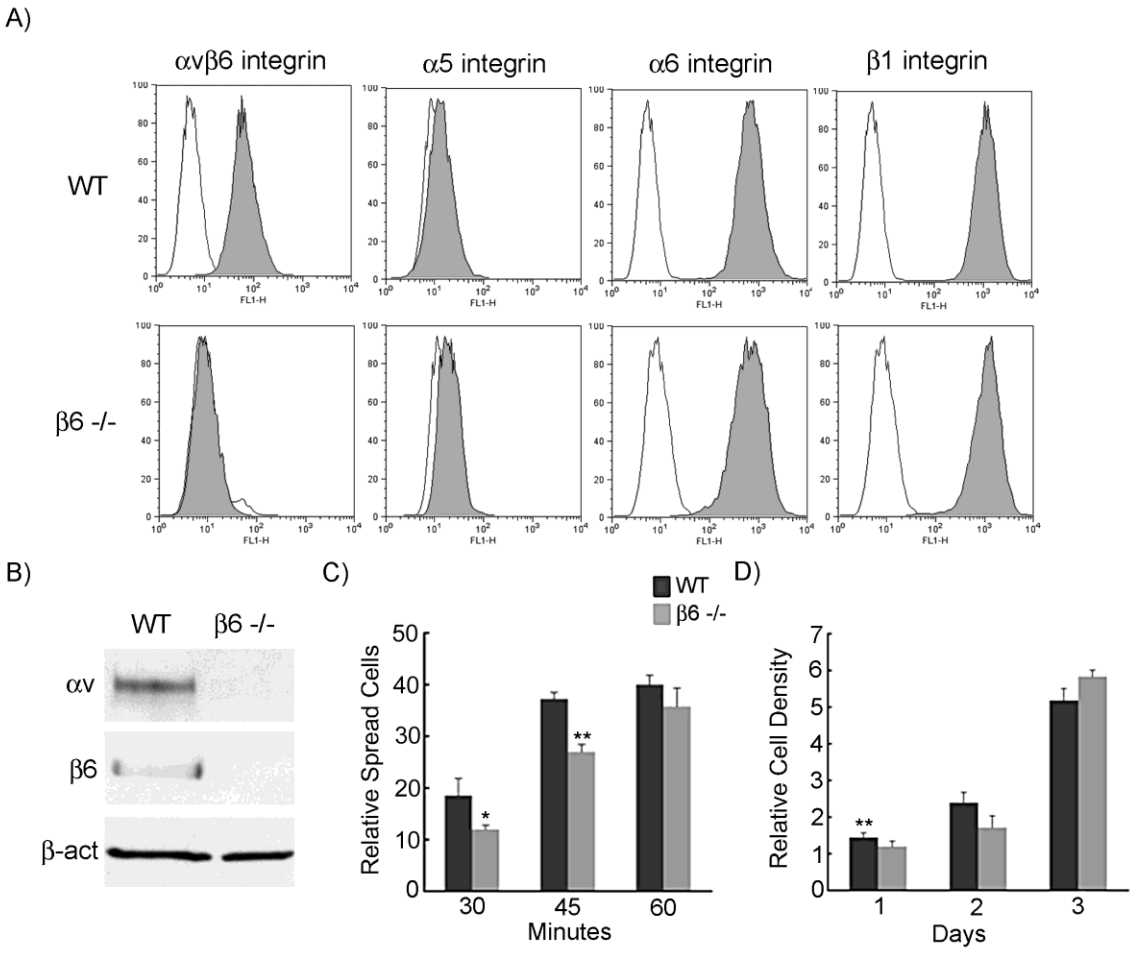

A)
αvβ6 integrin
α5 integrin
α6 integrin
β1 integrin
WT
β6 -/-
FL1-H
B)
WT
β6 -/-
αv
β6
β-act
C)
WT
β6 -/-
Relative Spread Cells
Minutes
30
45
60
D)
Relative Cell Density
Days
1
2
3

Figure 3.5. Characterization of progenitor-like mouse keratinocytes. A) The cell surface expression of α5, α6, and β1 integrin subunits and the αvβ6 integrin complex was analyzed in two WT and two β6-/- mouse keratinocyte cell lines by flow cytometry (tinted histograms). Cells immunostained without primary antibody were used as negative controls (white histograms). The experiment was repeated three times. Representative histograms are presented for one WT and one β6-/- cell line. The expression levels in the other two cell lines were similar to the ones presented. B) The expression of αv and β6 integrin subunits was also evaluated in WT and β6-/- mouse keratinocytes by Western blotting. C) WT and β6-/- mouse keratinocytes were compared in terms of their spreading on fibronectin. Cells were seeded on fibronectin-coated wells in serum-free conditions for 30, 45, and 60 minutes. At each indicated time point, cells were fixed with 4% (v/v) formaldehyde, and cell spreading was evaluated in four to six randomly selected fields by phase contrast microscope with X10 objective. Collagen-coated wells were served as control (not shown). The data shown here are the mean (± SD) of triplicate wells from a representative experiment. D) WT and β6-/- mouse keratinocytes were compared for their growth rate using Promega proliferation assay during a 5-day experiment. Cells were grown in keratinocyte complete medium for 18 hours (day-1) followed by terminating the cultures on days 2, 3, and 4. The data are shown as ratios of each indicated time point to day-1 value. The data shown here are the mean (± SD) of triplicate wells from a representative experiment.

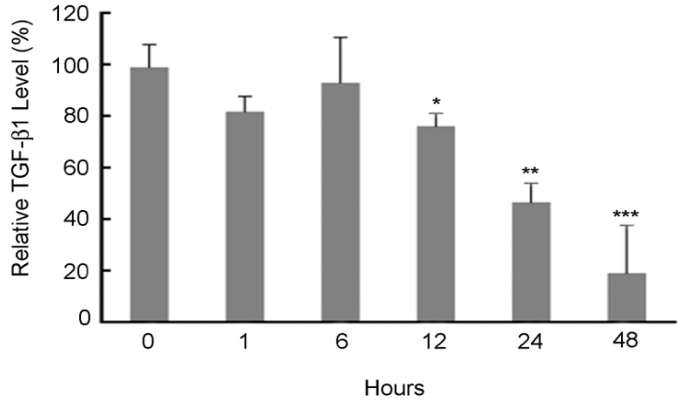
3.3.6. The β6-/- mouse keratinocytes do not remove latent TGF-β1 from the extracellular matrix

Our findings had shown that αvβ6 integrin regulates TGF-β decay from the LTBP-1-rich matrices in human HaCaT keratinocytes. Therefore, we next measured the level of matrix bound latent TGF-β1 in the cultures containing αvβ6 integrin expressing WT mouse keratinocytes seeded on LTBP-1-rich matrices. In the presence of the WT keratinocytes, latent TGF-β1started to decay from the matrix by 12 hours and continued to be removed until the end of the experiment at 48 h (Figure 3.6.A). Next, we tested the β6-/- mouse keratinocytes in a similar experiment at 24 h time point. Confirming our previous results, the WT keratinocytes removed significantly more latent TGF-β1 from the matrix as compared to the αvβ6 integrin-deficient cells (Figure 3.6.B).

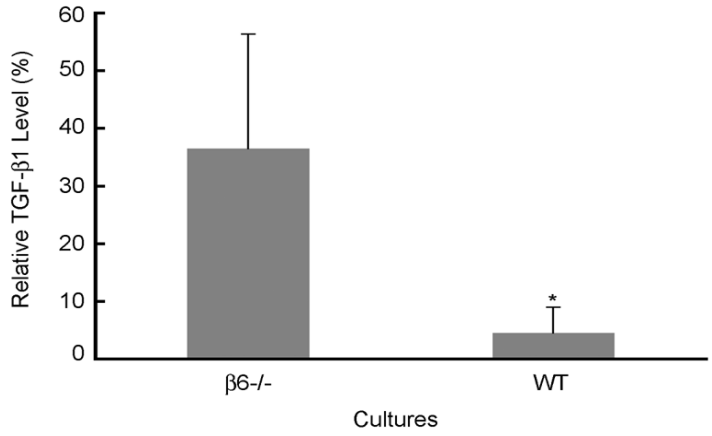
3.3.7. Cell proliferation of mouse keratinocytes on latent TGF-β1-rich extracellular matrix

One of the main biological effects of TGF-β1 in keratinocytes is growth inhibition (Sheppard, 2006). Therefore, we investigated whether high level of latent TGF-β1 deposited in LTBP-1-rich matrices differently affected the growth of WT compared to β6-/- mouse keratinocytes. Interestingly, growth of β6-/- cells was completely arrested on these matrices (Figure 3.6.C). The WT keratinocytes that were able to remove latent TGF-β1 from the matrix were, however, able to proliferate on these matrices (Figure 3.6.C).

A)


Relative TGF-β1 Level (%)
120
100
80
60
40
20
0
*
**
***
0
1
6
12
24
48
Hours

B)


Relative TGF-β1 Level (%)
60
50
40
30
20
10
0
*
β6-/-
WT
Cultures

C)

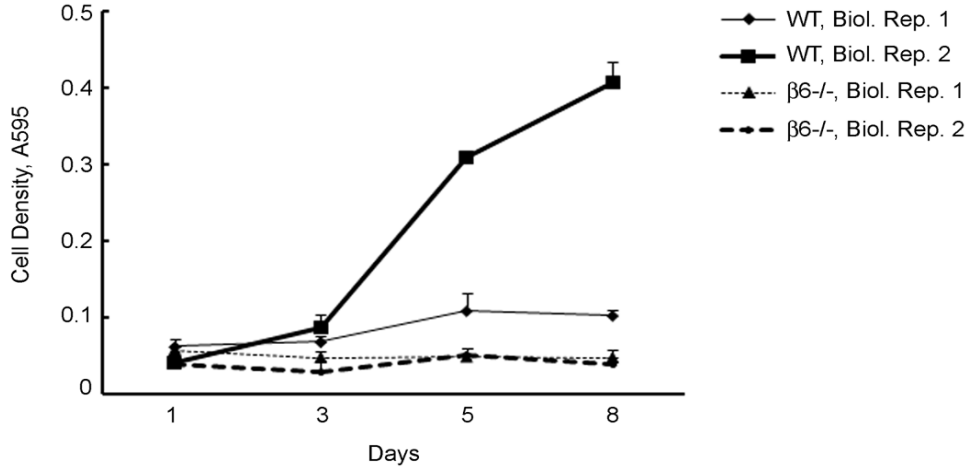
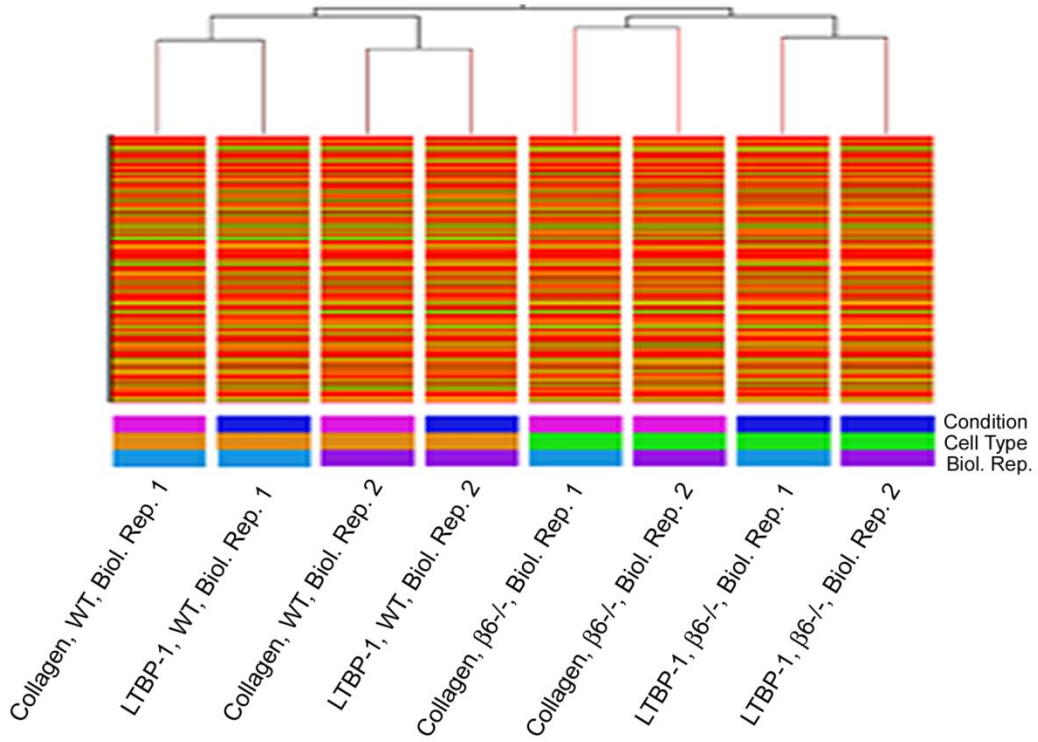

Cell Density, A595
0.5
0.4
0.3
0.2
0.1
0
1
3
5
8
Days
WT, Biol. Rep. 1
WT, Biol. Rep. 2
β6-/-, Biol. Rep. 1
β6-/-, Biol. Rep. 2

Figure 3.6. Behavior of wild-type versus β6-/- mouse keratinocytes on LTBP-1-rich matrix. A) Level of latent TGF-β1 was measured in the matrix portion of the cultures containing wild-type (WT) mouse keratinocytes seeded on LTBP-1-rich matrices in different time points from 0 to 48 hours, by ELISA. All values were normalized to the counterpart values of "LTBP-1-rich matrix only" samples and compared to "0 hour" time point for statistically significant differences (N=3). B) Level of latent TGF-β1 was measured by ELISA in the matrix portion of the cultures containing wild-type (WT) or β6-/- mouse keratinocytes seeded on LTBP-1-rich matrix. Cells were kept in culture in complete keratinocytes medium for overnight followed by a 24 hours serum deprivation. The values were normalized to the counterpart "LTBP-1-rich matrix only" samples (N=4 separate experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). C) Two biological replicates of wild-type and β6-/- mouse keratinocytes seeded on LTBP-1-rich matrix were compared for their growth rate using Promega proliferation assay during an 8-day experiment. Cells were grown in complete keratinocyte medium for 1, 3, 5, and 8 days before terminating the cultures. The data are shown as optical densities representing the cell number in each time point. The experiment was repeated three times. The data shown here are the mean (± SD) of triplicate wells from a representative experiment.

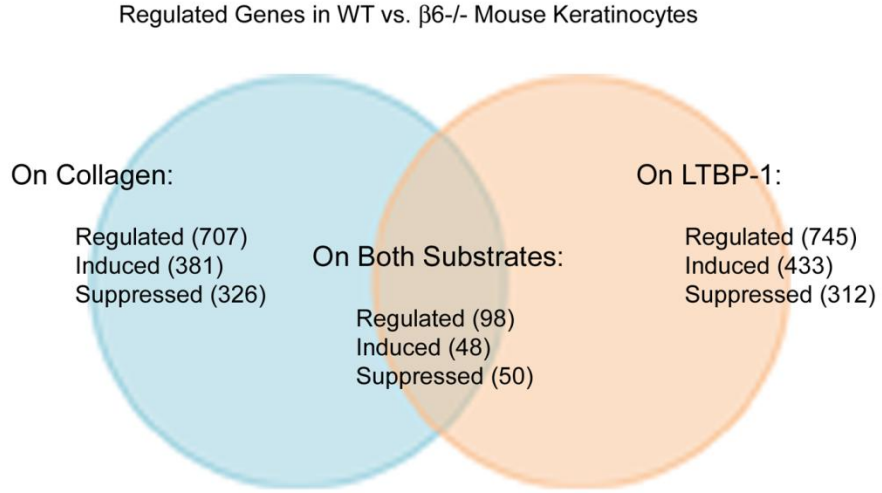
3.3.8. Regulation of gene expression by αvβ6 integrin in keratinocytes

To further investigate how αvβ6 integrin regulates the expression of genes in keratinocytes that are exposed to matrix-bound TGF-β1, gene expression profiling was performed using the Agilent whole mouse genome microarray kit. Two biological replicates of WT or β6-/- mouse keratinocytes were seeded on LTBP-1-rich matrices or collagen type-I (as the control substrate), generating eight experimental groups. Two-dimensional hierarchical clustering was performed to study relationships (shown by the dendrogram) among those experimental groups (Figure 3.7.A). Biological replicates of each cell type, WT or β6-/- mouse keratinocytes, showed high similarities in the pattern of altered gene expression. Although gene regulation in WT mouse keratinocytes was not dependent on the substrate that they were seeded on, biological replicates of β6-/- mouse keratinocytes showed distinct pattern of altered gene expression on LTBP-1-rich matrix versus collagen type I (Figure 3.7.A). Further comparisons were made between the average data generated from biological replicates of each cell type, WT or β6-/- mouse keratinocytes, seeded on LTBP-1-rich matrices or type I collagen. The presented Venn diagram (Figure 3.7.B) compares the number of genes relatively regulated in WT versus β6-/- cells seeded on either substrate. There were 745 genes regulated in the group seeded on LTBP-1-rich matrix, 707 genes in the group seeded on collagen type I, and only 98 genes common in both groups (Figure 3.7.B). Functional annotation of the differentially expressed genes in the cells seeded on LTBP-1-rich matrix was performed by the Ingenuity Pathway Analysis (IPA) software. For the WT mouse keratinocytes, IPA showed a predominant association of regulated genes with cell cycle, cellular growth, proliferation, and cancer (Figure 3.7.C). Next, potential relationship of the genes modulated by keratinocyte αvβ6 integrin was studied in WT versus β6-/- mouse keratinocytes seeded on LTBP-1-rich matrix by virtual regulatory network analysis using the IPA software (Figure 3.7.D). The highest scoring regulatory networks generated by IPA software in those groups were cell cycle-related networks, where many of the up-regulated genes in those networks had positive effects on cell cycle progression in the WT mouse keratinocytes, but not in their β6-/- counterparts (Table 3.2). Expression of cyclin B1 was confirmed to be elevated in the WT mouse keratinocytes compared to β6-/- cells by real-time PCR (not shown).

A)


WT vs. β6-/- Mouse Keratinocytes
Condition
Cell Type
Biol. Rep.
Collagen, WT, Biol. Rep. 1
LTBP-1, WT, Biol. Rep. 1
Collagen, WT, Biol. Rep. 2
LTBP-1, WT, Biol. Rep. 2
Collagen, β6-/-, Biol. Rep. 1
Collagen, β6-/-, Biol. Rep. 2
LTBP-1, β6-/-, Biol. Rep. 1
LTBP-1, β6-/-, Biol. Rep. 2

B)


Regulated Genes in WT vs. β6-/- Mouse Keratinocytes
On Collagen:
Regulated (707)
Induced (381)
Suppressed (326)
On Both Substrates:
Regulated (98)
Induced (48)
Suppressed (50)
On LTBP-1:
Regulated (745)
Induced (433)
Suppressed (312)

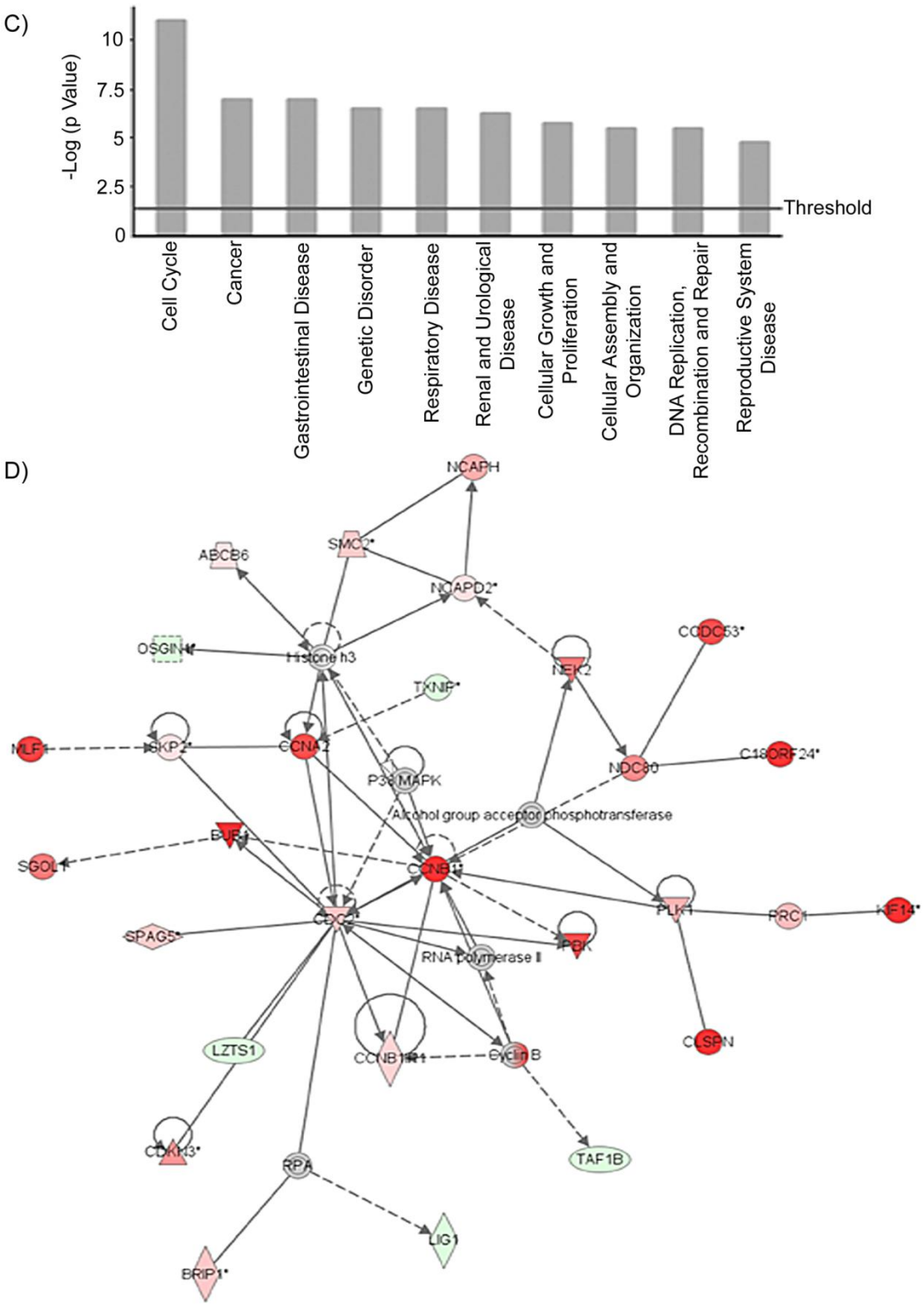

C)
-Log (p Value)
10
7.5
5
2.5
0
Threshold
Cell Cycle
Cancer
Gastrointestinal Disease
Genetic Disorder
Respiratory Disease
Renal and Urological Disease
Cellular Growth and Proliferation
Cellular Assembly and Organization
DNA Replication, Recombination and Repair
Reproductive System Disease
D)
NCAPH
SMC2
ABCB6
NCAPD2
CCDC53
OSGIN1
Histone h3
NEK2
TXNIP
MLF1
SKP2
CCNA2
C18ORF24
NDC80
P38 MAPK
Alcohol group acceptor phosphotransferase
BUB1
SGOL1
CCNB1
PLK1
PRC1
KIF14
CDC2
SPAG5
PBK
RNA polymerase II
CLSPN
LZTS1
CCNB1IP1
Cyclin B
CDKN3
TAF1B
RPA
LIG1
BRIP1

Figure 3.7. Transcriptional profiling of the genes regulated in wild-type (WT) versus β6-/- mouse keratinocytes; A) Pattern of gene expression and modulation in wild-type and β6-/- mouse keratinocytes seeded on latent TGF-β1 (LTBP-1) -rich matrices or type I collagen at twofold or greater variation between groups and *p* value < 0.05 for significance. There were eight experimental groups representing two biological replicates (Biol. Rep.) from each cell line seeded on either matrix (LTBP-1 or Collagen). Each column represents the normalized probe set signal intensity values for a single experimental group. B) Venn diagram of the regulated gene lists. The presented Venn diagram gives an overview of the regulated genes (≥ 2-fold variation between groups, *p* value < 0.05), induced or suppressed, in the WT versus β6-/- mouse keratinocytes seeded on LTBP-1-rich matrices or collagen type I. C) Functional annotation of αvβ6-dependent genes regulated in wild-type versus β6-/- mouse keratinocytes exposed to matrix-bound latent TGF-β1. The gene lists used for IPA were the the originally selected regulated genes in the wild-type versus β6-/- cells seeded on LTBP-1-rich matrix at twofold or greater change from the baseline and *p* value less than 0.05 for significance. D) Network analysis of genes differentially regulated in wild-type versus β6-/- mouse keratinocytes seeded on LTBP-1-rich matrix. Shown is the highest scoring regulatory network selected from the gene lists that were also used for functional annotation. Regulated genes are colored by red (expressed more in the WT cells) for induced and green for suppressed genes. The color intensity varies with detected signal intensity. Uncolored symbols represent the genes that were not regulated in the presented gene lists but served as links between different parts of the network. The complete name of the regulated genes in the network can be found in Table-2.

Table 3.2. List of the regulated genes involved in cell cycle-related network generated by IPA software in the presence of wild-type mouse keratinocytes seeded on LTBP-1-rich matrix when compared to β6-/- counterparts. Results are presented at the fold changes ≥ 2 or ≤ 0.5, and *p* value < 0.05.

Gene Symbol	Fold Change	*p* Value	Description	Accession Number
Kif14	84.6	0.00223	Mus musculus kinesin family member 14	BC054123
Clspn	49.25	0.0149	Mus musculus claspin homolog (Xenopus laevis)	NM_175554
Ccnb1	31.6	0.0116	Mus musculus cyclin B1	NM_172301
Ccnb1	24.77	0.0129	Mus musculus cyclin B1	NM_172301
Mlf1	22.33	0.00339	Mus musculus myeloid leukemia factor 1	NM_010801
Ccna2	21.08	0.00843	Mus musculus cyclin A2	NM_009828
Pbk	21.05	0.000876	Mus musculus PDZ binding kinase	NM_023209
Sgol1	15.79	0.0159	Mus musculus shugoshin-like 1 (S. pombe)	NM_028232
Nek2	15.61	0.0039	Mus musculus NIMA-related expressed kinase 2	NM_010892

Gene Symbol	Fold Change	*p* Value	Description	Accession Number
Kntc2	13.76	0.00249	Mus musculus kinetochore associated 2	NM_023294
Cdkn3	12.45	0.0277	Mus musculus cyclin-dependent kinase inhibitor 3	BC049694
Bub1b	12.28	0.0164	Mus musculus budding uninhibited by benzimidazoles 1, beta	NM_009773
Brrn1	9.771	0.02	Mus musculus barren homolog (Drosophila)	NM_144818
Plk1	9.285	0.0188	Mus musculus polo-like kinase 1 (Drosophila)	NM_011121
Prc1	8.05	0.00542	Mus musculus protein regulator of cytokinesis 1	NM_145150
Cdc2a	6.897	0.0184	Mus musculus cell division cycle 2 homolog A (S. pombe)	NM_007659
Brip1	6.522	0.00552	Mus musculus BRCA1 interacting protein C-terminal helicase 1	NM_178309
Spag5	6.369	0.0455	Mus musculus sperm associated antigen 5	NM_017407
Smc2l1	6.289	0.0295	Mus musculus structural maintenance of chromosomes 2-like 1	NM_008017
Smc2l1	4.048	0.0257	Mus musculus structural maintenance of chromosomes 2-like 1	NM_008017

Gene Symbol	Fold Change	*p* Value	Description	Accession Number
Smc2l1	3.496	0.0233	Mus musculus structural maintenance of chromosomes 2-like 1	NM_008017
Abcb6	3.177	0.0147	Mus musculus ATP-binding cassette, sub-family B, member 6	NM_023732
Skp2	2.06	0.0409	Mus musculus SCF complex protein Skp2	AF083215
Txnip	0.465	0.0401	Mus musculus thioredoxin interacting protein , transcript variant 2	NM_023719
Taf1b	0.319	0.0076	Mus musculus TATA box binding pr-associated factor, RNA polymerase I- B	NM_020614
Lig1	0.314	0.0205	Mus musculus ligase I, DNA, ATP-dependent	NM_010715
Txnip	0.304	0.0321	Mus musculus thioredoxin interacting protein, transcript variant 2	NM_023719

3.4. Discussion

In the present study, we found that endogenous and matrix-bound TGF-βs are differentially regulated by αvβ6 integrin in keratinocytes. Autogenic TGF-β1 regulated its target genes, including αvβ6 integrin, while extracellular matrix-bound latent TGF-β was effectively removed from the matrix by αvβ6 integrin, allowing cells to continue to proliferate. We have shown previously that the expression of αvβ6 integrin is drastically regulated in keratinocytes by TGF-β1 (Koivisto et al., 1999). In addition, the data of the present report and our previously published observations (Ghannad et al., 2008) support the notion that endogenous TGF-β1 production and activation plays an important role in de novo synthesis of αvβ6 integrin in keratinocytes. This effect seems rather specific to TGF-β1 as other cytokines do not seem to significantly regulate β6 integrin expression in cultured keratinocytes (Ghannad et al., 2008). Similar results have been published using cytomegalovirus-infected endothelial cells in which both TGF-β neutralizing antibody or signaling inhibitor strongly suppressed αvβ6 integrin expression (Tabata et al., 2008). In keratinocytes, blocking αvβ6 integrin or neutralizing TGF-β1 leads to reduced expression of CTGF (connective tissue growth factor) that mediates expression of TGF-β-regulated ECM genes. These observations suggest that significant level of regulation by autogenic TGF-β1 is mediated via αvβ6 integrin in keratinocytes. These findings are in agreement with the early studies with other cell systems demonstrating that αvβ6 integrin can activate TGF-β1 (Munger et al., 1999). Interestingly, blocking αvβ6 integrin also led to the up-regulation of other genes involved in TGF-β activation and signaling, including TGF-β1, TGF-β3, SARA, and smad2, most probably as a compensatory effect to the lack of TGF-β1 activation. Therefore, it is conceivable that αvβ6 integrin also reciprocally regulates the endogenous TGF-β production in keratinocytes. Consistent with these findings, up-regulation of TGF-β1 gene expression has also been shown in smad4-deficient skin and cutaneous wounds with reduced level of TGF-β signaling (Owens et al., 2009). The simplest explanation for the ineffectiveness of blocking β6 integrin on the smad signaling or expression of TGF-β1 targets on the LTBP-1 matrix is that some of the matrix bound TGF-β1 is in active form that renders β6 integrin blocking useless. Using the ELISA assays, however, we were unable to find significant amount of active TGF-β1 in the matrix. Although we cannot entirely exclude this possibility, it is

feasible that αvβ6 integrin-independent activation mechanisms are also involved in regulating the smad signaling pathway.

We found that keratinocytes seeded on LTBP-1-rich matrix depleted TGF-β1 from the matrix. Depletion of matrix-bound latent TGF-β1 by keratinocytes was αvβ6 integrin-dependent as well as cell number-dependent, suggesting that cell-to-cell contacts could be necessary for the decay of TGF-β1 from matrix. This is not surprising, as activation of TGF-β1 by αvβ6 integrin has been shown to be cell-to-cell contact-dependent (Munger et al., 1999). It is likely that decay of matrix-associated latent TGF-β1 initially follows the same mechanism as the activation process in which αvβ6 integrin first binds to the LAP of the complex, leading to a conformational change that subsequently releases active TGF-β1 to its receptor (Annes et al., 2004). It has been shown that TGF-β together with its receptor complex becomes internalized in order to go through either signaling or degradation pathways inside the cells (Hoeller et al., 2005). Internalization of αvβ6 integrin together with a number of its ligands, including LAP (as LAP-Fc), has also been reported before (Berryman et al., 2005; Weinreb et al., 2004). We found that treating HaCaT keratinocytes with LAP-Fc resulted in localization of αvβ6 integrin into focal adhesions and also in the intracellular vesicles. This finding should be further confirmed using recombinant human LAP since it may behave differently than LAP conjugated to Fc that contained a point mutation in LAP cDNA (Weinreb et al., 2004). Further studies are required to study the possible localization of the β6 integrin as well as LAP to the markers of endocytotic pathways in the presence or absence of the β6 function-blocking antibody, SLC, and LLC in order to have a better understanding of the role of the β6 integrin in binding to and possibly internalizing a part or the whole complex of TGF-β. Attempts to chemically block the endocytosis pathways proved difficult, as keratinocytes were extremely sensitive to these compounds. Therefore, we can only speculate that in the presence of high levels of latent TGF-β1, some may get activated by αvβ6 integrin but get directed to degradation rather than signaling and some may become endocytosed with the integrin itself. Future studies need to investigate these speculations further.

One of the most significant functions of TGF-β1 is in regulation of cell proliferation. Inhibition of epithelial cell proliferation by TGF-β1 involves downregulation of c-Myc, leading

to the upregulation of cyclin-dependent kinase inhibitors p15 and p21, which inhibit the CDK4/6-cyclin D and CDK2-cyclin E-mediated phosphorylation of the retinoblastoma protein (Robson et al., 1999; Ten Dijke et al., 2002). Blocking TGF-β1 signaling during keratinocyte migration appears to improve wound re-epithelialization by removing TGF-β-mediated inhibition of cell proliferation (Amendt et al., 2002; Ashcroft et al., 1999). Interestingly, αvβ6 integrin does not seem to have a functional role in wound healing in mouse models, unless the animals are challenged, suggesting that αvβ6 integrin-mediated TGF-β1 activation plays a lesser role in early re-epithelialization of wound healing likely because of redundant activation mechanisms present in early wounds (Häkkinen et al., 2004; Xie et al., 2009). When mouse wound healing is challenged by corticosteroids, however, αvβ6 integrin appears to gain its function and control of cell proliferation through TGF-β1 activation during the re-epithelialization phase (Xie et al., 2009). It is worthwhile to note that in human wounds both αvβ6 integrin and TGF-β1 protein levels peak when re-epithelialization is complete (Eslami et al., 2009; Haapasalmi et al., 1996; Häkkinen et al., 2000; Honardoust et al., 2008). At the time of wound closure, keratinocytes typically show increased proliferation rate in spite of high level of TGF-β1 in the matrix surrounding them. It is possible, therefore, that in this context, αvβ6 integrin could protect keratinocytes from the growth inhibition by TGF-β1 by degradation (decay) of excessive TGF-β1 levels. In support of this hypothesis, wild-type β6+/+ mouse keratinocytes showed a progressive growth rate on the LTBP-1-rich matrix, while the β6-/- keratinocytes were growth arrested. In keeping with the results of the growth assay, our transcriptional profiling demonstrated that, compared to the β6-/- cells, the wild-type mouse keratinocytes seeded on LTBP-1-rich matrix differentially expressed many genes that have positive effects on the cell cycle progression.

In summary, we have shown that keratinocytes differentially regulate endogenous and matrix bound latent TGF-β1. We hypothesize that the αvβ6 integrin-dependent decay of matrix bound latent TGF-β1 is a potential mechanism by which keratinocytes manage high levels of extracellular TGF-β1 possibly through internalization of αvβ6 integrin – latent TGF-β1 complexes.

3.5. References

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Chapter 4: General Discussion

Despite induction of the αvβ6 integrin expression in skin and mucosal wound keratinocytes, the role of this integrin during wound healing remains largely unknown. Interestingly, β6 gene deletion does not disturb normal wound closure in mice possibly due to redundant mechanisms of TGF-β activation present in early stages of wound healing (Häkkinen et al., 2004; Xie et al., 2009). It is also possible that TGF-β1 activation is not needed for early wound re-epithelialization or it may even have negative effect. An overexpression of TGF-β1 in epidermis in mice has been associated with a decreased keratinocyte proliferation rate and delayed re-epithelialization in burn wounds (Yang et al., 2001). Moreover, it has been shown that blocking TGF-β signaling improved full-thickness skin wound re-epithelialization by removing TGF-β-mediated inhibition of cell proliferation (Amendt et al., 2002; Ashcroft et al., 1999; Huang et al., 2002). All of these studies showed that suppression of TGF-β1 activation or signaling speeds up re-epithelialization in wounds that lack a dermal substrate as a support for keratinocyte migration (full-thickness or burn wounds) by increasing keratinocyte proliferation rate and, therefore, resulting in faster wound closure. When wound healing is compromised, however, TGF-β1 activation may play a more significant role. In old β6 integrin deficient mice, wound healing is delayed mainly during granulation tissue formation stage (Aldahlawi et al., 2006). This may relate to suboptimal TGF-β activation by αvβ6 integrin as TGF-β levels drop with aging (Schmid et al., 1993; Ashcroft et al., 1997; Wu et al., 1999). It has also been shown that the elimination of αvβ6 integrin speeds up re-epithelialization in immunodeficient mice through decreasing the activity of TGF-β1 and removing the inhibitory effects of TGF-β1 on keratinocyte proliferation (Xie et al., 2009). The enhanced proliferation in these mice was seen especially in hair follicles that are known to contribute to dermal wound healing (Argyris, 1976; Taylor et al., 2000; Ito et al., 2005; Levy et al., 2007; Xie et al., 2009). Taken together, early re-epithelialization does not seem to be dependent on either TGF-β or expression of αvβ6 integrin unless healing is challenged. It appears, therefore, that the role of αvβ6 integrin in early wound healing is in controlling proliferation via TGF-β activation rather than supporting epithelial migration that it can do *in vitro* (Huang et al., 1998; Koivisto et al., 1999, 2006).

In previous studies, the peak expression of the αvβ6 integrin has been shown to occur around day 7 post-wounding when re-epithelialization is complete (Haapasalmi et al., 1996; Häkkinen et al., 2000; Honardoust et al., 2008). In the present thesis, we showed that αvβ6 integrin is persistently expressed and accumulated in scarless gingival wounds with a peak between 7 to 28 days post-wounding. In scar-forming skin wounds, however, αvβ6 integrin peaked at 7 days post-wounding and was downregulated soon after. At its peak expression, the αvβ6 integrin highly co-accumulated with TGF-β1 and TGF-β3 in human gingival wounds. The co-accumulation of the αvβ6 integrin with TGF-β1 and TGF-β3 was also observed at certain time points of gingival and skin wounds in pigs. These data suggest a more important role for αvβ6 integrin in wound resolution, which occurs at later stages of wound healing, rather than during keratinocyte migration and wound closure. As an activator of TGF-β1 and TGF-β3, it is possible that αvβ6 integrin modulates locally the activation of TGF-β isoforms that are important regulators of wound healing. Once the wound is completely covered by epithelial cells, TGF-β1 could regulate keratinocyte proliferation, inflammation, or granulation tissue formation (or all of them).

Activation of TGF-β via αvβ6 integrin has been reported to require cell-cell contacts, LTBP-1 and fibronectin (Munger et al., 1999; Annes et al., 2004; Fontana et al., 2005). Furthermore, cells need intact actin cytoskeleton for pulling the latent complex to open up the TGF-β complex for presentation of active TGF-β to the TGF-β receptor II (Munger et al., 1999). Potentially, the latent TGF-β complex could signal through the αvβ6 integrin during this process as modulation of actin cytoskeleton is known to induce cell signaling (Zhao et al., 2007; Moustakas and Heldin, 2008; Huveneers and Danen, 2009; Olson and Nordheim, 2010). Integrin-mediated signaling during the activation process remains unknown, however. We have demonstrated that αvβ6 integrin accumulates together with TGF-β1 and TGF-β3 at the basal cell layer of the wound epithelium where they co-localize (Eslami et al., 2009). Although not experimentally shown, the receptor and ligand are likely to be close enough for activation to happen in the basal layer. In addition, cell-cell contacts are present at this location. It is more problematic to speculate how the rest of the activation of TGF-β via αvβ6 integrin could happen. First, at the time of the co-localization of the ligand and receptor, the basement membrane with hemidesmosomal attachments has formed (Kirfel and Herzog, 2004). It is not easy to understand

how basal cells could initiate mechanical forces in the focal actin adhesions between the firm points of hemidesmosomes and activate integrin bound TGF-β1 latent complex. In addition, among the two additional proteins required for activation, fibronectin is not present at the lamina lucida of the basement membranes (Uitto and Pulkkinen, 1996) and precise location of LTBP-1 has not been reported. We have immunolocalized LTBP-1 in gingival wounds and it seems to localize to the basement membrane zone (Eslami et al., 2009). It is conceivable; therefore, that complementary or compensatory proteins and mechanisms are involved in TGF-β1 activation by αvβ6 at the basal cell layer after basement membrane formation. Fibrillin-1 could act as one such protein. It is synthesized by both keratinocytes and fibroblasts and can affect the activation of TGF-β1 through controlling its bioavailability (Kielty et al., 1993; Taipale et al., 1995; Haynes et al., 1997; Chaudhry et al., 2007). Exact localization of fibrillin in the basal cell layer remains to be investigated. In summary, integrin mediated activation of TGF-β1 has been proven both *in vitro* and *in vivo* (Yang et al., 2007; Wipff and Hinz, 2008; Aluwihare et al., 2009). In addition, epithelial αvβ6 integrin-mediated activation of TGF-β1 in many fibrotic animal models has been demonstrated. However, the activation mechanism in the presence of basement membrane needs to be further investigated.

As per some previous studies, we also showed that at the time of wound closure, keratinocytes normally show an increased proliferation rate regardless of high levels of TGF-β1 in their surrounding matrix (Clark, 1996; Schäfer and Werner, 2007; Honardoust et al., 2008; Eslami et al., 2009). The results from our *in vitro* studies on the cultured keratinocytes suggest an explanation for this discrepancy and propose a novel biologic role for the αvβ6 integrin in regulating TGF-β1 homeostasis. Although our results suggested that keratinocyte αvβ6 integrin activates endogenously produced TGF-β1, it was found that higher levels of matrix-bound TGF-β1 were eliminated by this integrin. This allowed the cells to continue proliferation even in the presence of TGF-β1. This may explain how wound keratinocytes modulate the excessive presence of TGF-β1 (with its growth inhibitory effects) in their extracellular matrix when they must proliferate at the time of wound closure. Further experiments are, however, required to confirm these findings. In summary, considering a possible scavenging role for the αvβ6 integrin in removing the excess of matrix-bound TGF-β1 from the cell environment at the time of wound

closure could potentially be a natural survival tool by which cells could reduce the outcomes of TGF-β1 activation during the proliferative phase of wound re-epithelialization.

TGF-β1 is a powerful anti-inflammatory molecule as discussed in the section 1.5.1. Therefore, resolution of inflammation by TGF-β1 may also play a role in distinguishing healing outcomes between gingiva and skin. Assuming that the main function of αvβ6 in the wound epithelium is the activation of TGF-βs, we could speculate that longer expression of αvβ6 in the gingiva could prolong the presence of diffusible paracrine active TGF-β1 that could reduce inflammation in the gingiva faster than in the skin. In agreement with this speculation are the observations that inflammation is linked to scar formation and scarless healing in the gingiva is associated with faster resolution of inflammation (Mak et al., 2009). Furthermore, prolonged expression of αvβ6 integrin and its co-accumulation with anti-fibrotic TGF-β3 in gingival wounds but not in skin wounds, may at least in part contribute to the scarless phenotype of gingiva. Our previous unpublished data show that keratinocytes and fibroblasts may have direct connections through cell processes when basement membrane is restored during wound healing. This kind of keratinocyte-fibroblast connection may explain how activation of TGF-β isoforms by keratinocyte αvβ6 integrin may contribute to the wound healing outcome in terms of scar formation. Further investigations are required to show such possibilities.

4.1. Limitations

In our descriptive study of human gingival wounds in search for a potential for the αvβ6 integrin-mediated regulation of TGF-βs, we were lucky enough to have healthy volunteers to go through the wounding procedure and sampling. However, we were not able to study the parallel skin wound healing in humans in the same way due to a lack of donors. Red Duroc pigs were, therefore, chosen for a comparative study of scarless gingival versus scar-forming skin wound healing in terms of the co-accumulation and expression of the αvβ6 integrin as well as TGF-β1 and TGF-β3. Here we proposed that αvβ6 integrin could potentially but differentially regulate TGF-β1 and TGF-β3 during scarless gingival versus scar-forming skin wound healing. Such a different regulation of TGF-β isoforms may determine the wound outcome to heal normally or produce scar. This hypothesis should be further investigated through functional studies in suitable animal models, such as red Duroc pigs, through studying the impact of local blockade of

the αvβ6 integrin on TGF-β activation, cell proliferation and scar formation in different time points of gingival versus skin wound healing.

In our *in vitro* cell experiments we had to indirectly assess the activation of endogenously produced versus matrix-bound TGF-β by keratinocyte αvβ6 integrin through studying the expression of TGF-β-induced target genes. Using TGF-β bioassay to directly detect active TGF-β in the cell cultures was not possible since in the single cultures of reporter cells (mink lung epithelial cells or MLECs) high levels of active TGF-β was detected. This high background from the reporter cells alone made it very difficult to interpret the results from co-cultures of keratinocytes and reporter cells in different treatment settings. It is possible that the presence of very small amount of active TGF-β in LTBP-1-rich matrix (as shown by our ELISA results – unpublished) is enough to result in high response from MLECs. Pre-treatment of LTBP-1-rich matrix with LAP before populating them with cells may neutralize the active portion of TGF-β in the matrix and result in more meaningful results. It is also possible that there may be activators of TGF-β other than αvβ6 integrin in the cultures containing MLECs. This possibility, however, has not been supported by the existing literature nor by our unpublished data using specific inhibitors against proteases involved in TGF-β activation.

LTBP-1-rich matrix has been previously used to study the mechanism of αvβ6 integrin-mediated activation of TGF-β that requires counter-traction forces from both the ECM and the cell cytoskeleton. The composition of extracellular matrix (ECM) is an important factor that could support the transmission of mechanical forces from cell cytoskeleton to the matrix. For example, the presence of fibronectin (FN) in the ECM has been shown to promote LTBP-1 incorporation into the ECM and the αvβ6 integrin-mediated activation of TGF-β (Fontana et al., 2005). We could not detect FN in the LTBP-1-rich matrix produced by CHO cells possibly because our antibodies against FN did not recognize the FN produced by hamster. However, defining the components of LTBP-1-rich matrix seems to be a necessary step to be performed in characterizing this substrate.

To study the mechanism of the αvβ6 integrin-mediated decay of TGF-β1 from LTBP-1-rich matrix, we attempted to chemically block the main endocytosis pathways mediated through either clathrin pit or caveola formation. These experiments proved difficult since the inhibitors of

endocytosis were extremely toxic for keratinocytes. Our findings showed that treating HaCaT keratinocytes with Fc-labeled LAP led to the localization of the αvβ6 integrin into focal adhesions and also in the intracellular vesicles. Further studies are required to study the possible localization of the β6 integrin as well as LAP to the markers of endocytotic pathways in the presence or absence of the β6 function-blocking antibody, SLC, and LLC in order to have a better understanding of the role of the β6 integrin in binding to and possibly internalizing a part or the whole complex of TGF-β.

In our *in vitro* studies, we focused on investigating the regulatory role of the αvβ6 integrin on TGF-β1 for two reasons. The first reason was the importance of TGF-β1 as the most frequently expressed isoform of TGF-β in many physiological and pathological conditions including normal and abnormal wound healing. The second reason was the availability of CHO cell-produced matrix rich in latent TGF-β1 as an excellent substrate to study the αvβ6 integrin-mediated regulation of matrix-bound TGF-β1. Future studies should, however, investigate the regulatory mechanisms of TGF-β3 by the αvβ6 integrin since those mechanisms may be applicable in the local regulation of TGF-β3 with its anti-scarring effects during wound healing.

4.2. Future direction

In this dissertation, we showed that there is a potential for the αvβ6 integrin-mediated regulation of TGF-β1 and TGF-β3 during wound healing. We also demonstrated an extended expression of the αvβ6 integrin and anti-fibrotic TGF-β3 in scarless gingival wounds as compared to skin wounds by immunolocalization and gene expression studies. This may, at least in part, contribute to the scarless phenotype of gingival wounds. To further pursue this possibility, we propose an *in vivo* experiment in red Duroc pigs to study how inhibiting β6 integrin in different time points of gingival wound healing may change the wound outcome. We anticipate that systemic application of small molecule αvβ6 integrin inhibitor (EMD527040, Popv et al., 2008) in later stages of gingival wound healing, when αvβ6 integrin and TGF-β3 are highly co-localized, may initiate fibrotic changes that correlate with scar formation. Moreover, it is not known how the αvβ6 integrin-mediated activation of TGF-βs affects wound outcomes including scar formation in the presence of a newly formed basement membrane. We

hypothesize that keratinocytes and fibroblasts have direct connections through cell processes when basement membrane is restored during wound healing. This kind of keratinocyte-fibroblast connection may explain how activation of TGF-β isoforms by keratinocyte αvβ6 integrin could contribute to the wound healing outcome in terms of scar formation.

Based on the *in vitro* cell experiments, we proposed that the keratinocyte αvβ6 integrin may have a dual role in activating endogenously produced TGF-β1 while removing matrix-bound TGF-β1, thus protecting the cells from TGF-β1-mediated growth inhibitory effects. The details of the mechanisms involved in the αvβ6 integrin-mediated decay of matrix-bound TGF-β1 (including the internalization pathways involved) should be further investigated. Attempts to chemically block the endocytosis pathways proved difficult, as keratinocytes were extremely sensitive to these compounds. Alternatively, endocytosis can be inhibited by applying small interfering RNA (siRNA) silencing of specific markers of internalization pathways known to be used by αvβ6 integrin (*e.g.*, clathrin and β2- microglobulin, Berryman et al., 2005; Heikkilä et al., 2010). Subsequently, by immunoflouresecence staining and confocal microscopy, localization of β6 integrin together with LAP (as part of latent TGF-β) can be studied in keratinocytes seeded on LTBP-1-rich matrix through a time-course experiment in the presence or absence of β6 function-blocking antibodies. Our hypothesis is that the αvβ6 integrin-dependent decay of matrix bound latent TGF-β1 is a potential mechanism by which keratinocytes manage high levels of extracellular TGF-β1 possibly through internalization of "αvβ6 integrin / latent TGF-β1" complexes.

Meanwhile, when planning to use therapeutic targeting of the αvβ6 integrin to prevent or improve fibrotic conditions mediated by TGF-β1 over-activity, the novel role of the αvβ6 integrin in removing the excess of TGF-β1 from the cell environment should also be considered.

4.3. References

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Appendix A: Ethics Certificate

The University of British Columbia
Office of Research Services
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver, BC V5Z 1L8

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: RENEWAL

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC CREB NUMBER:
Hannu S. Larjava		H05-70585

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
UBC	Vancouver (excludes UBC Hospital)

Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):

Lari T. Hakkinen

SPONSORING AGENCIES:

- Canadian Institutes of Health Research (CIHR) - "Function of Keratinocyte Alphavbeta6 Integrin in Wound Healing and Function of Small Leucine-Rich Proteoglycans in Wound Healing (CIHR MOP-12589 and 77550) (Gene Expression During Scarless Gingival Wound Healing)"
- Canadian Institutes of Health Research (CIHR) - "Mechanisms of re-epithelialization: The role of glycogen synthase kinase-3"

PROJECT TITLE:
Function of Keratinocyte Alphavbeta6 Integrin in Wound Healing and Function of Small Leucine-Rich Proteoglycans in Wound Healing (CIHR MOP-12589 and 77550) (Gene Expression During Scarless Gingival Wound Healing)
Mechanism of re-epithelialization: The role of glycogen synthase kinase-3

EXPIRY DATE OF THIS APPROVAL: December 4, 2010

APPROVAL DATE: December 4, 2009

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Clinical Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Clinical Research Ethics Board.

Approval of the Clinical Research Ethics Board by one of:

Dr. Peter Loewen, Chair
Dr. James McCormack, Associate Chair